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UNIVERSITY OF ALBERTA

Characterization of the Tellurite Resistance Determinants of the IncPα Plasmid RK2 and the IncHII Plasmid pHH1508a

by

EMILY G. WALTER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND

RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MEDICAL MICROBIOLOGY AND INFECTIOUS DISEASES

EDMONTON, ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Characterization of the Tellurite Resistance Determinants of the IncPα Plasmid RK2 and the IncHII Plasmid pHH1508a submitted by Emily G. Walter in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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This thesis is dedicated to the memory of my father, Frank Alexander Grant, whose encouragement and enthusiasm started me on this path and whose moral and scientific example have guided me ever since.

Abstract

Resistance to toxic tellurium compounds (Te^R) in the *Enterobacteriaceae* is predominantly mediated by H and P incompatibility group plasmids. The Te^R determinants from the IncHII plasmid pHH1508a and the broad host range IncPα plasmid RK2 were cloned and sequenced. The Te^R genes of RK2 were located between the *kilA* and *korA* genes involved in plasmid replication control. Transposon mutagenesis and RNA primer extension suggested the *kilA* and Te^R genes are transcribed from a common promoter. The open reading frame (ORF) for *kilA* was identified in the nucleotide sequence as well as two ORFs in the Te^R region which were named *telA* and *telB*. Their gene products have predicted sizes of 28, 42, and 32 kilodaltons, respectively. All three polypeptides were detected after overproduction in a T7 RNA polymerase/promoter system.

The DNA sequence of the Te^R determinant of pHH1508a was determined and two ORFs, named *tehA* and *tehB*, were identified. These proteins were overproduced and had molecular masses of 28 and 23 kDa, respectively, as determined by their electrophoretic migration in SDS polyacrylamide gels. The size of TehA predicted from the nucleotide sequence was 36 kDa. Anomalous migration of this protein may be due to its highly hydrophobic sequence.

Construction of alkaline phosphatase and β-galactosidase fusions to the *telB* and *tehA* gene products suggested these

proteins are located in the inner membrane of the bacterium. This was supported by hydrophobicity plot analysis which predicted hydrophobic membrane-spanning regions in both proteins.

Media-conditioning experiments showed that *E. coli* (pDT1364 [HII Te^R]) but not *E. coli* (pDT1558 [Pα Te^R]) reduced the toxicity towards Te^S *E. coli* of broth containing potassium tellurite by at least 64-fold. Similarly, pDT1364 but not pDT1558 increased the rate of reduction of tellurite to metallic tellurium by *E. coli*. These results suggest that the mechanism of Te^R mediated by pHH1508a is detoxification via a "tellurite reductase". The plasmid RK2Te^R appears to encode a different mechanism.

Analysis of these genes will help to determine the mechanism of bacterial resistance to tellurium compounds and its importance to the maintenance of these plasmids in environmental and clinical bacterial isolates.

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Abbreviations

A	Adenine
aa	Amino acid(s)*
ATP	Adenosine triphosphate
BHI	Brain heart infusion
BPB	Bromophenol blue
C	Cytosine
CsCl	Cesium chloride
DNA	Deoxyribonucleic acid
DNP	2,4-dinitrophenol
DNAase	Deoxyribonuclease
EDTA	Disodium ethylene diamine tetraacetate
EtBr	Ethidium bromide
FAD	Flavin adenine dinucleotide
G	Guanine
h	hour(s)
Inc	Incompatibility group
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	kilobase
KCN	potassium cyanide
kDa	kilodalton
LacZ	β-Galactosidase
LB	Luria broth
M	Molar

*Individual amino acid abbreviations are listed in Appendix 2.

min.	minute(s)
MIC	Minimum Inhibitory Concentration
ml	Millilitre
mRNA	Messenger RNA
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
nt	Nucleotide(s)
NTP	Nucleoside triphosphate
OD	Optical density
O/N	Overnight
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PhoA	Alkaline phosphatase
RBS	Ribosome binding site
RNA	Ribonucleic acid
RNAase	Ribonuclease
RT	Room temperature
rpm	Revolutions per minute
SD	Shine-Dalgarno Sequence
SDS	Sodium dodecyl sulphate
T	Thymine
Te	Tellurite
TE	Tris EDTA buffer
Tn	Transposon
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet

XGal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XP	5-bromo-4-chloro-3-indolyl-phosphate

1. Introduction

1.1 Bacterial resistance to metals.

Bacteria grow in the presence of a wide variety of ions and metals, many of which are essential for bacterial cell growth and division. These include compounds of cobalt, copper, iron, magnesium, manganese, molybdenum, phosphorous, potassium, sodium, sulfur, zinc, etc. (reviewed by Hughes and Poole, 1989). The levels of these compounds within the cell are carefully regulated by a number of specific ion transport systems. Too high concentrations of nutrient metals or the presence of low levels of other non-nutrient metals can be toxic to the bacterial cell. Toxic metals are often able to enter the cell via the normal nutrient transport systems. Once inside the cell, they can exert their toxic effects. In order to survive in the presence of high levels of these toxic metals, bacteria have evolved ways of preventing the internal concentration of the noxious compound from rising to lethal levels. The mechanisms used are often similar to those used in resistance to antibiotics (Foster, 1983). These include (a) entry exclusion, for example, in some of the mechanisms of chloramphenicol and aminoglycoside resistance (Bryan et al., 1976; Nicas and Hancock, 1980; Bryan et al., 1984; Burns et al., 1985); (b) extrusion, eg. tetracycline resistance (McMurry et al., 1980); (c) detoxification, eg. β-lactam resistance (Abraham and Chain,

1940); and (d) target modification, eg. macrolide-lincosamide-streptogramin B (MLS) resistance (Lai et al., 1973). This last mechanism has not yet been encountered in microbial resistance to metals, probably because of the more generalized nature of the targets of metal toxicity (Summers and Barkay, 1989).

Many bacterial strains are able to grow in the presence of high concentrations of compounds of toxic metals including antimony, arsenic, cadmium, chromium, cobalt, copper, lead, mercury, nickel, selenium, silver, tellurium, and zinc (Summers, 1986; Silver and Misra, 1988). This resistance is frequently encoded by extrachromosomal, self-replicating molecules of DNA called plasmids (Smith, 1967; Novick and Roth, 1968). Some large plasmids such as the F factor (100 kilobases (kb)) encode proteins that enable the transfer of the plasmid between bacterial cells via conjugative pili on the surface of the bacterium (Willetts and Skurray, 1987). This transfer of plasmids allows rapid spread of resistance determinants between bacterial strains and even species, either in the environment or within the human body. Some antibiotic and metal resistance determinants are encoded on transposons. These are mobile units of DNA which are able to mediate their own transfer from one location on the chromosome or plasmid to another within the bacterial cell (Craig and Kleckner, 1987).

The frequency of occurrence of different metal resistances in bacteria in environmental and clinical

isolates has been studied for comparison with the frequency of antibiotic resistance. Resistance to arsenic, cadmium, mercury, and tellurium are among the most common (Nakahara et al., 1977; Marques et al., 1979; Harnett and Gyles, 1984). These are often encoded by plasmids which also carry determinants of resistance to antibiotics used to treat either human or animal diseases. Thus, both types of resistance may be selected for simultaneously in a hospital setting. In addition, industrial and agricultural pollution of rivers and soils may contribute to the selection for metal-resistant, and thus antibiotic-resistant, bacteria in the environment. Considerable research is currently underway to utilize bacterial metal resistances for the recovery of commercially valuable metals such as silver from the environment and forthe treatment of polluted sewages (Mergeay, 1990).

Mercury Resistance. One of the most well-characterized metal resistances is mercury resistance. Mercury compounds have been used clinically in the treatment of syphilis, in diuretics and in hospital antiseptics and disinfectants (Webb, 1966; Petering and Tepper, 1976; Foye, 1977; Porter et al., 1982). Currently, mercury compounds are found in dental amalgam and the release of these compounds may select for mercury resistant bacteria in the gastrointestinal tract of humans (Summers, A. O., Seminar, Am. Soc. Microb. 1990). Mercury compounds are toxic to bacteria because of their

ability to bind to sulfhydryl groups, thereby inhibiting enzyme action (Fildes, 1940; Webb, 1966).

Gram-positive and gram-negative bacteria encode similar mechanisms of resistance to mercury ions. The transposon *Tn21*, located on the gram-negative bacterial (*Shigella*) plasmid R100, encodes the *merTPCAD* operon (de la Cruz and Grinsted, 1982; Ni'Bhriain et al., 1983; Barrineau et al., 1984; Ni'Bhriain and Foster, 1986). This operon is both positively and negatively regulated at the level of transcription by the product of the divergently transcribed *merR* gene (Foster et al., 1979; Foster and Brown, 1985; Foster and Ginnity, 1985; Lund et al., 1986). MerR binds to the *mer* operator-promoter (*mer-op*) region probably by means of a helix-turn-helix motif characteristic of DNA binding proteins (Pabo and Sauer, 1984; Heltzel et al., 1987; O'Halloran and Walsh, 1987; Ross et al., 1989). MerR binds to *mer-op* as a dimer both in the presence and absence of mercury (O'Halloran and Walsh, 1987). Upon binding of Hg^{2+} by cysteine residues in the carboxy-terminus of MerR, the dimer activates transcription of the *mer* operon through interactions with both RNA polymerase and *mer-op* (Shewchuk et al., 1989a,b,c; O'Halloran et al., 1989). The exact function of MerD is unknown, however, it may also be involved in regulation (Nucifora et al., 1989b).

MerT (located in the bacterial inner membrane) and MerP (a periplasmic Hg^{2+}-binding protein) form a mercury uptake system which channels the Hg^{2+} ions to the cytoplasmic

mercuric reductase formed by MerA (Jackson and Summers, 1982a, 1982b; Ni' Bhriain and Foster, 1986; Lund and Brown, 1987). This mercury transport system was identified through the isolation of mutants in *merA* which were hypersensitive to mercury (Nakahara et al., 1979). MerC, which shares more than 45 % homology with MerT, may also be involved in mercury transport (Summers, 1986; Kusano et al., 1990). Hg^{2+} is detoxified by MerA through conversion to the volatile, less toxic metal (Hg^0) using NADPH (Fox and Walsh, 1982, 1983). In addition to FAD, three cysteine residues are located within the active sites of MerA which are involved in binding and reduction of the mercury ion (Schultz et al., 1985; Distefano et al., 1989; Miller et al., 1989; Moore and Walsh, 1989). Mercuric reductase has considerable homology to other NADPH-dependent oxido-reductases including glutathione reductase and lipoamide dehydrogenase (Fox and Walsh, 1983; Misra et al., 1985).

Arsenic Resistance. Arsenic compounds have also been used in the treatment of syphilis (Eagle and Doak, 1951; Foye, 1977) and, until recently, in the treatment of certain protozoal diseases (Klevay, 1976a). Bacterial cells take up arsenate ions by the phosphate transport systems (Willsky and Malamy, 1980). Arsenic is toxic to bacteria because of its ability to inhibit thiol-containing enzymes and by acting as a phosphate analogue (Klein and Boyer, 1972; Albert, 1973; Summers and Silver, 1978). Inducible resistance to arsenate,

arsenite and antimony (III) is mediated by plasmids in both *Staphylococcus aureus* and *Escherichia coli*. Arsenate-resistant stains of *S. aureus* have been isolated from victims of toxic shock syndrome (Barbour, 1981).

The *ars* operon of the plasmid R773 in *E. coli* encodes an ATP-dependent efflux system which has homology to other ATP-binding proteins and to mammalian multidrug resistance glycoproteins (Mobley and Rosen, 1982; Chen et al., 1986; Rosen et al., 1988; Silver and Misra, 1988). Resistance to arsenite in *Alcaligenes* may be due to the presence of an intracellular enzyme which oxidizes arsenite to the less toxic arsenate (Legge, 1954; Ehrlich, 1978; Osborne and Ehrlich, 1976; Phillips and Taylor, 1976).

Cadmium Resistance. Cadmium is an important environmental pollutant. Cadmium ions enter gram-positive bacterial cells by the manganese transport system and gram-negative bacterial cells by the zinc transport system (Tynecka et al., 1981a; Laddaga and Silver, 1985). They are toxic to bacteria because of their ability to compete with zinc and inhibit sulfhydryl groups in proteins; they can also induce single strand breaks in DNA to create mutations (Vallee and Ulmer, 1972; Mitra and Bernstein, 1978; Summers, 1984).

Several plasmid-determined cadmium resistance determinants have been characterized. The *cadA* gene on *S. aureus* plasmids specifies a cadmium ion translocating ATPase (Tynecka et al., 1981b; Witte et al., 1986; Silver et al.,

1989; Nucifora et al., 1989a). This gene has 25 % homology to K^+ ATPases from *E. coli* and *Streptococcus faecalis* and weaker homology to the Ca^{2+} ATPase of the sarcoplasmic reticulum and Na^+/K^+ exchange ATPases of animal cell membranes and the fungal cell membrane proton ATPases (Silver and Misra, 1988). The CadA protein has homology to MerA and MerP in its amino-terminus and in its possession of two cysteine residues which may be involved in metal binding (Silver and Misra, 1988; Nucifora et al., 1989a).

The *Alcaligenes eutrophus* plasmid operon (Czc) confers simultaneous resistance to cadmium, zinc and cobalt by means of an inducible energy-dependent efflux system which is unrelated to *cadA* (Nies et al., 1989a; Nies and Silver, 1989).

The *cadB* determinant of *S. aureus* plasmid pI258 appears to encode the production of a Cd^{2+}-binding protein (Perry and Silver, 1982). Another cadmium-binding protein has also been reported which appears to have homology to the metallothioneins of animal cells (Olafson, 1984; Higham et al., 1984; Silver and Misra, 1988). These are cysteine-rich polypeptides which protect eukaryotic cells from toxic metals by binding metal cations (Hamer, 1986). Finally, chromosomal mutations in *Bacillus subtilis* resulting in a change in the manganese transport system can result in cadmium resistance (Laddaga et al., 1985).

Chromate Resistance. Chromate (CrO_4^{2-}) ions are taken up by the sulfate transport system (Ohtake et al., 1987). A chromate resistance determinant carried on a plasmid isolated from *Pseudomonas aeruginosa* has recently been cloned and sequenced (Cervantes et al., 1990). This determinant causes a decreased accumulation of chromate in *P. aeruginosa* cells (Cervantes and Ohtake, 1988). This is due to a single highly hydrophobic protein, ChrA, which has 29 % amino acid homology with another chromate resistance determinant from *A. eutrophus* (Nies et al., 1989b; Nies et al., 1990).

In *Enterobacter cloacae*, chromate reductase activities have been detected which are associated with the membrane fraction of cells, and may be responsible for resistance and the ability to use chromate as an electron acceptor under anaerobic conditions (Wang, et al., 1989, 1990).

Copper Resistance. Copper compounds have been used as antibacterial agents in the protection of plants and as growth stimulants for pigs (Foyé, 1977; Tetaz and Luke, 1983). At least three different copper resistance mechanisms have been identified. Resistance determined by plasmid pRJ1004 in *E. coli* is inducible (Tetaz and Luke, 1983; Rouch et al., 1985). Through interactions with two chromosomal loci, the plasmid *pco* genes regulate the intracellular level of copper by influencing its uptake, storage, and efflux (Summers and Barkay, 1989; Rouch et al., 1989; Mergeay, 1990). In the plant pathogen *Pseudomonas syringae*, copper

binding proteins appear to mediate resistance to copper (Silver and Misra, 1988). Copper resistance in *Mycobacterium scrofulaceum* appears to be due to the intracellular precipitation of copper sulfide (Erardi et al., 1987).

Silver Resistance. Compounds containing silver are used as prophylactic agents in the prevention of gonorrheal ophthalmia neonatorum and as topical antiseptics for the treatment of severe burns (Foye, 1977; Summers et al., 1978). Silver has a wide range of toxic effects on bacteria. It precipitates bacterial proteins, DNA, and RNA, and it interferes with respiration and other cell surface-associated functions (Rosenkranz and Carr, 1972; Bragg and Rainnie, 1974; Foye, 1977). Silver-resistant bacteria are occasionally isolated from burn wounds of patients but they are more commonly found in industrial sludge (Summers et al., 1978). The mechanism of plasmid-encoded silver resistance has not yet been determined, however, chromosomal resistance in *E. coli* is due to a decrease in silver binding by a major outer membrane protein (Pugsley and Schnaitman, 1978).

Selenium Resistance. Selenium is an essential trace element (Stadtman, 1974) and it is found in some bacterial enzymes such as formic dehydrogenase in *E. coli* (Pinsent, 1954). Selenium compounds are toxic to most microorganisms probably due to their strong oxidizing ability and the ability of selenium to act as a sulfur analogue (Scala and

Williams, 1962). Selenite has been used in biological media for the selection of *Shigella* and *Salmonella*, which are naturally resistant (Shrift and Boulette, 1974; Klevay, 1976b).

Many bacteria can either methylate or reduce selenium-containing compounds (Chau et al., 1976; Doran and Alexander, 1977; Summers and Silver, 1978). Selenite- and selenium-reducing activities have been found in *Micrococcus lactolyticus* (Woolfolk and Whiteley, 1962), *Streptococcus* (Tilton et al., 1967a, 1967b), *Salmonella* (McCready et al., 1966) and *Clostridium* genera (Garcia and Bush, 1989). Selenium granules are accumulated in the cell membrane and cell wall in some bacteria (Gerrard et al., 1974) and in the cytoplasm in others (Silverberg et al., 1976). It was suggested that this reduction of selenite to red elemental selenium was not the mechanism of resistance since the accumulation of these selenium granules causes damage to the cell, probably due to disruption of cellular integrity (McCready et al., 1966; Ahluwalia et al., 1968; Konetzka, 1977). However, plasmid-mediated resistance to selenite in *Pseudomonas* sp. was found to be associated with the reduction of selenite to elemental selenium (Jackson et al., 1988; Latinwo et al., 1990). In addition, a strain of *Pseudomonas* was recently found to be able to grow under anaerobic conditions using selenate as an electron acceptor and this was associated with reduction of selenate to selenium (Macy and Rech, 1990; Steinberg et al., 1990).

1.2 Bacterial resistance to tellurite.

Occurrence of tellurium. Tellurium (atomic number 52) is a semi-metal which belongs to the same group in the periodic table as selenium and sulfur. Tellurium compounds are found in low concentrations in the environment and in moderate amounts in foods such as garlic (Schroeder et al., 1967). In addition, fairly large amounts are found in the human body (Schroeder et al., 1967; Nason and Schroeder, 1967).

Tellurium compounds are by-products of copper, nickel, silver, lead, and gold refining processes (Klevay, 1976c). Tellurium is used as a catalyst in the film industry, in the manufacture of batteries, alloys, rubber, and as a coloring agent in glass (Browning, 1969).

Recently, an immuno-modulating compound containing tellurium has been tested in rats for possible use in treating AIDS and cancer patients (Sredni et al., 1987; Nyska et al., 1989). This compound, called AS-101, stimulates lymphoid cells to proliferate and produce interleukin 2 *in vitro*, apparently by increasing the intracellular calcium concentration. The effect may also be partly due to a direct stimulatory effect on the immune system by tellurium (Nyska et al., 1989).

Toxicity. Tellurium is moderately toxic to humans. One of the most obvious symptoms of tellurium poisoning is the characteristic garlic odor on the breath. This appears to be

due to the production of methylated forms of tellurite, such as volatile dimethyl telluride (Cerwenka and Cooper, 1961). A garlic odor is also observed when either resistant or sensitive strains of *P. aeruginosa* are grown on a sub-inhibitory concentration of tellurite (Summers and Jacoby, 1977). In contrast, *E. coli* strains do not produce this odor. The production of dimethyltelluride by a *Penicillium* fungus has been demonstrated, however, this occurred only in the presence of both tellurium and selenium (Fleming and Alexander, 1972).

Tellurite is toxic to most microorganisms, particularly gram-negative bacteria, possibly due to its strong oxidizing ability (Fleming 1932; Scala and Williams, 1963; Summers and Jacoby, 1977). Tellurite anions (TeO_3^{2-}) have been found to be more toxic than tellurate anions (TeO_4^{2-}) (Scala and Williams, 1963). The sensitivity of *E. coli* bacterial cells to tellurite and selenite was found to be enhanced by the presence of L-methionine (Scala and Williams, 1962, 1963). Since tellurite and selenite are chemically similar to sulfate, these authors proposed that tellurite and selenite could be reduced, and thus detoxified, by the sulfate-reduction pathway; the presence of an exogenous reduced sulfur source such as methionine would repress this pathway, thus decreasing the rate of detoxification of selenite and tellurite, and therefore increasing sensitivity to these anions.

Bacterial modification of tellurite. Bacteria grown on media containing potassium tellurite (K_2TeO_3) form black colonies. Electron microscopy, X-ray diffraction, and electron spectroscopic imaging (ESI) have shown that these bacteria contain black intracellular crystals of metallic tellurium which are often located just inside the inner membrane (Tucker et al., 1962; Taylor et al., 1988). Sensitive bacteria can also form black colonies when grown at sub-inhibitory concentrations of tellurite (Summers and Jacoby, 1977). The ability of potassium tellurite to be reduced to black metallic tellurium has been used in the identification of reductive sites in bacterial and eukaryotic cells. Barrnett and Palade (1957) and Wachstein (1949) used tellurite reduction by respiratory enzyme systems to localize these enzymes within the cell to the mitochondria by electron microscopy. In gram-positive and gram-negative bacteria, tellurium was found to be predominantly associated with the membranes (Morton and Anderson, 1941; Mudd et al., 1956; Iterson and Leene, 1964a,b). This observation was used to suggest that the respiratory enzyme system of bacteria is located in the cytoplasmic membrane.

Resistance. Some gram-positive bacteria, including *Corynebacterium diphtheriae* (Conradi and Troch, 1912), *Streptococcus faecalis* (now called *Enterococcus faecalis*) (Skadhauge, 1950; Appleman and Heinmiller, 1961), and most strains of *Staphylococcus aureus* (Hoeprich et al., 1960), are

naturally resistant to potassium tellurite. For this reason, tellurite media has long been used as a means of identification of *C. diphtheriae*. In addition, the ability of mycobacteria to produce black colonies when grown on media containing tellurite has been used to test for the viability of the tubercle bacilli (Corper, 1915; Kilburn et al., 1969). The resistance of *Alcaligenes* to tellurite is used to differentiate *A. faecalis* and *A. denitrificans* from *Bordetella bronchiseptica* (Johnson and Sneath, 1973). Some coliform bacteria, which are extremely sensitive to tellurite, can be acclimatised to higher concentrations by passage in tellurite broth (Fleming and Young, 1940).

Escherichia coli appears to take up tellurite by one of the phosphate transport systems (Tomás and Kay, 1986). Spontaneous mutants of *E. coli* could be obtained which were resistant to low levels of tellurite (~10 µg/ml) as well as to arsenate. These mutants were found to be defective in phosphate transport and were unable to grow on media containing low levels of phosphate. Transport of phosphate was competitively inhibited by tellurite. Susceptibility to tellurite could be restored by a plasmid carrying the *phoB* region which is involved in phosphate regulation.

Thermus thermophilus and *T. flavus* are extremely thermophilic gram-negative rods which are resistant to many heavy metal salts and toxic ions (Chiong et al., 1988a). During studies aimed at finding an easily selectable marker to use in genetic experiments within this genus, Chiong and

coworkers found that both of these species of bacteria are resistant to potassium tellurite as well as sodium selenite, and produce a strong garlic odor when grown in the presence of tellurite (Chiong et al., 1988a). This resistance did not appear to be plasmid-mediated. Several different bacterial cell fractions were obtained which had tellurite-reducing activity (Chiong et al., 1988b). A protein of approximately 53 kilodaltons (kDa) was purified and characterized from one of the fractions. The enzyme catalyzed the reduction of potassium tellurite to tellurium in the presence of NADH or NADPH. In addition, this enzyme was able to reduce sodium selenite and sodium sulfite. This group hypothesized that tellurite resistance in *Thermus* spp. is due to the presence of these tellurite reductases.

Cell-free extracts of *Mycobacterium avium* are also capable of reducing potassium tellurite in the presence of NADH or malate and malic dehydrogenase (Terai et al., 1958). Woolfolk and Whiteley (1962) found that extracts of *Micrococcus lactilyticus* reduce tellurium compounds at the expense of molecular hydrogen. However, they were unable to detect the reduction of tellurite with molecular hydrogen using extracts of *E. coli*.

Gram-negative tellurite-resistant bacteria are frequently isolated from hospital and urban sewages, and film-reprocessing sludge (Taylor and Summers, 1979). In the Enterobacteriaceae, tellurite resistance is often mediated by plasmids of the H and P incompatibility groups (Summers and

Jacoby, 1977; Taylor and Summers, 1979; Bradley et al., 1982; Bradley, 1985).

1.3 Plasmids of the P incompatibility group.

Plasmids belonging to the same incompatibility group are unable to stably coexist in the same bacterial host (reviewed by Novick, 1987). This is usually because they share either replication control or partitioning functions. Plasmids of the P incompatibility group have the ability to transfer between, and replicate and be stably maintained in a wide variety of Gram-negative bacteria (for review see Thomas and Smith, 1987; Thomas, 1989). They are frequently found to encode antibiotic resistance in clinical isolates. IncP plasmids may be divided into IncPα and IncPβ subgroups based on homology in the origin of transfer (*oriT*) region and heteroduplex analysis (Yakobson and Guiney, 1983; Villarroel et al., 1983).

The P incompatibility group in *E. coli* is equivalent to the P-1 group in *P. aeruginosa*. The narrow host range P-2 group plasmids in *Pseudomonas* also express resistance to tellurite (Summers and Jacoby, 1977). In addition, these plasmids inhibit the replication of certain DNA phages (Jacoby et al., 1983).

The 60 kb plasmid RK2 is a member of the IncPα group. RK2 and the plasmids RP4, RP1, R18 and R68 were originally isolated from gram-negative bacteria obtained from a hospital

in Birmingham (U.K.) in 1969 (Holloway and Richmond, 1973; Ingram et al., 1973). They are indistinguishable by restriction endonuclease mapping and by heteroduplex analysis and therefore their names are used interchangeably (Burkardt et al., 1979; Currier and Morgan, 1981). RP4 has a G + C content of about 60 % (Holloway and Richmond, 1973), consistent with its isolation from *Pseudomonas* (66 % G + C; Marmur and Doty, 1962). It carries resistances to ampicillin, kanamycin, and tetracycline, and is reported to decrease the virulence of its host bacterium (Wretlind et al., 1985; Onaolapo and Klemperer, 1987).

The minimal replication system of RK2 includes *oriV* (origin of vegetative replication) and the *trfA* (trans-acting replication function) gene (Thomas et al., 1980; Schmidhauser and Helinski, 1985). The TrfA protein binds to a number of tandem repeats at the *oriV* region and is essential for *oriV* activation (Pinkney et al., 1988). A number of other determinants have been identified on this plasmid which are involved in plasmid stability in various bacterial hosts. These include *kil* genes which are potentially lethal to the *E. coli* host bacterium and *kor* or *kil*-override genes which inhibit the effect of *kil* genes on the cell (Figurski et al., 1982). Expression of the *kilA* gene is negatively regulated by *korA* and *korB* (Young et al., 1985; Young et al., 1987). The *kilA* gene maps to the 0' to 2.3' region on this plasmid (Figure 1; Young et al., 1985). The *korA* and *korB* genes are transcribed together in an operon

and have been located to the 56'-60' region on the map of RK2 (Smith and Thomas, 1984; Bechhofer et al., 1986). In addition to autoregulation of the *korA-korB* operon, KorA and KorB negatively regulate transcription initiation at the *trfA* operon probably by binding to inverted repeats in the operator region of this operon (Shingler and Thomas, 1984; Smith et al., 1984; Schreiner et al., 1985; Theophilus et al., 1985; Bechhofer et al., 1986; Thomas and Helinski, 1989).

When variants of RP4 are selected on media containing tellurite, they express resistance to tellurite (Bradley, 1985; Taylor and Bradley, 1987). Mutation to tellurite resistance (Te^R) appears to occur at a frequency of 1.7×10^{-8} (Bradley et al., 1988). No changes could be seen in the restriction maps which could account for the acquisition of Te^R (Taylor and Bradley, 1987). This cryptic Te^R determinant has been located between the *kilA* and *korA* genes using Tn7 insertion mutagenesis (Taylor and Bradley, 1987). It was found to be carried on a transposon, Tn*521*, which is about 4.5 kb in size and appears to include the *kilA* and *korA* genes (Bradley and Taylor, 1987; Bradley et al., 1988; Grewal, 1990). Transposition occurs at a very low frequency (Bradley et al., 1988). Tellurite resistance could be expressed in five different bacterial genera. No cross resistance to arsenic, antimony or selenium compounds was found (Bradley et al., 1988). IncPβ plasmids have large regions of homology

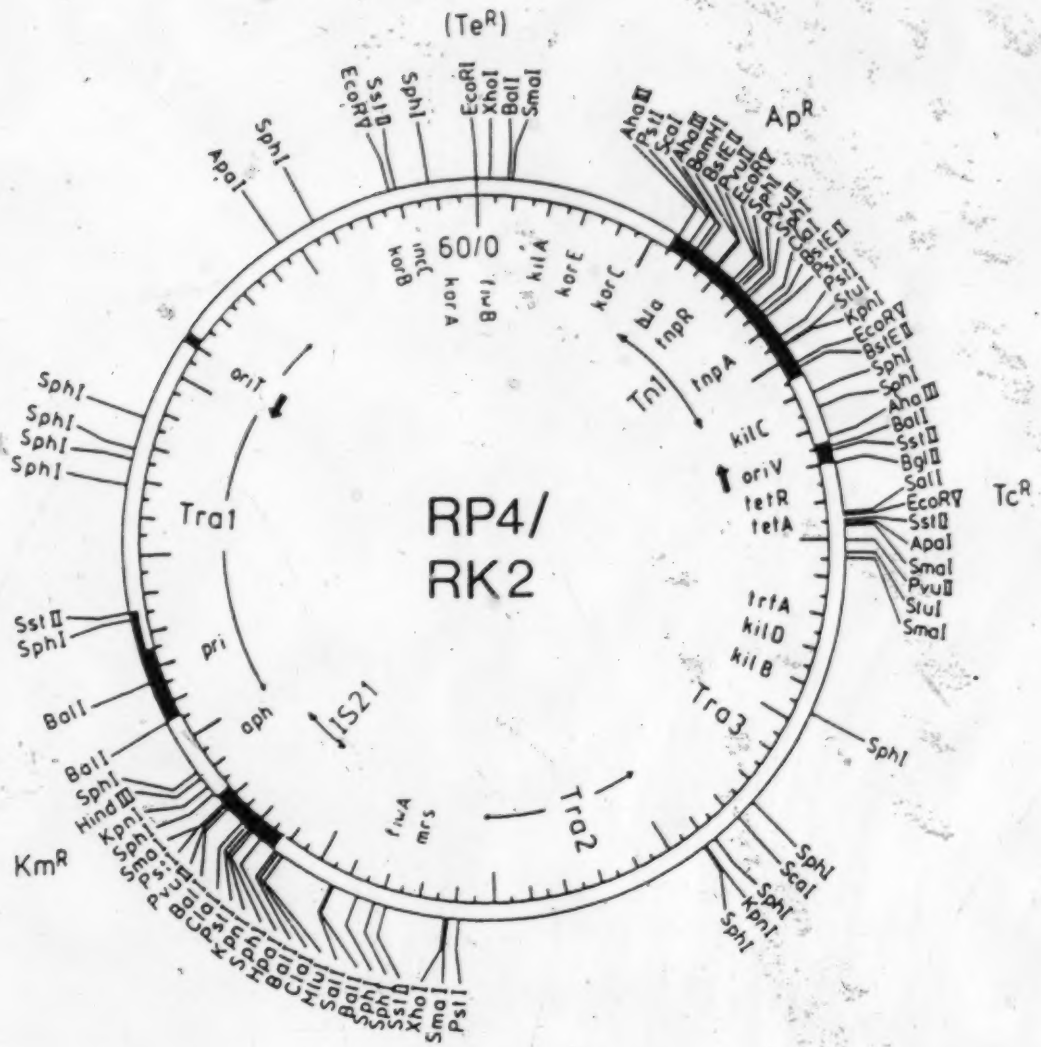


Figure 1. Restriction map of RP4/RK2.
Circular map of the broad host range IncPα plasmid RK2 (=RP4) showing the positions of various genes and restriction endonuclease sites on this plasmid (modified from Thomas and Smith, 1987).

with RK2; however, they do not carry a Te^R determinant (Villarroel et al., 1983; Bradley, 1985).

The plasmid RP1 is capable of inhibiting the fertility of the IncW plasmid R388 by a factor of 10^6 when both plasmids are present in the same host bacterium (Yusoff and Stanisich, 1984). Two functions involved in this property have been found on RP1 and named *fiwA* and *fiwB*. The *fiwB* function was mapped to the same region as the Te^R determinant (Fong and Stanisich, 1989). Insertions of Tn*504* which eliminated expression of this fertility inhibition function also eliminated expression of tellurite resistance.

1.4 Plasmids of the H incompatibility group.

Occurrence of H plasmids. The H plasmids are large (greater than 150 kb), conjugative plasmids that encode multiple drug resistances (Anderson and Smith, 1972; Anderson, 1975). They were first discovered to code for resistance to chloramphenicol in *Salmonella typhi* in an epidemic of typhoid fever in Mexico in 1972, and were responsible for antibiotic resistance in outbreaks of typhoid fever in India, Vietnam and Thailand (Anderson and Smith, 1972; Taylor et al., 1985b).

Tellurite-resistant bacteria carrying H plasmids are often isolated from film-reprocessing sludge as well as city and hospital sewages (Taylor and Summers, 1979). These plasmids frequently carry resistance to mercury as well.

Classification of H group plasmids. The H plasmids are divided into two incompatibility groups, IncHI and IncHII. The IncHI plasmids are thermosensitive in their conjugative transfer, in that transfer is more efficient at temperatures below 30°C (Anderson, 1975). They are further divided into three subgroups, HI1, HI2, and HI3, based on studies of the DNA-DNA homology and incompatibility properties (Grindley et al., 1972; Bradley et al., 1982; Roussel and Chabbert, 1978). Plasmids in each subgroup are strongly incompatibile with others from the same subgroup (Taylor and Grant, 1977). The unselected plasmid is rapidly lost and often plasmid recombination also occurs. Weaker incompatibility exists between the subgroups although plasmids within each subgroup have little homology with plasmids from the other two subgroups (Taylor and Grant, 1977; Whiteley and Taylor, 1983). IncHI1 plasmids also show one-way incompatibility with the F factor (Smith et al., 1973). Elimination of F by HI1 plasmids can be explained by the presence of homologous replication regions on both F and HI1 plasmids (Taylor et al., 1985c; Saul et al., 1988).

HI1 and HI2 plasmids specify thick, flexible pili which are serologically unrelated to other conjugative pili (Bradley et al., 1980). The presence of these pili allows adsorption and infection by a specific RNA bacteriophage called pilHα (Coetzee et al., 1985). A new group of plasmids encoding H pili constitutively was identified in 1982. However, these plasmids were compatible with, and did not

surface exclude, other HI plasmids (Bradley et al., 1982). Plasmids with these properties, including pHH1508a and pHH1457, were classified as IncHII plasmids. In contrast to those belonging to the IncHI group, these plasmids are not temperature-sensitive in their conjugative transfer (Bradley et al., 1982).

IncHI plasmids. R27 is the best studied of the IncHI1 group of plasmids. First isolated from *Salmonella typhimurium* in 1961, it has a size of 182 kb and carries resistance to tetracycline on a transposon, Tn*10* (Meynell and Datta, 1966; Grindley et al., 1972, 1973; Taylor and Brose, 1985). In addition, this plasmid enables its host to utilize citrate as its sole carbon source (Smith et al., 1978a; Taylor and Brose, 1986). In contrast to the F factor, whose conjugative transfer genes are well characterized and are grouped together in a single region (Willetts and Skurray, 1987), the transfer genes on R27 are located in at least two widely separated regions (Taylor et al., 1985a).

The IncHI2 plasmid, pMER610, was originally isolated from an *Alcaligenes* species of bacteria. It is a large (>250 kb) conjugative plasmid that encodes resistance to mercury and tellurium compounds and mediates phage inhibition (Jobling and Ritchie, 1987). The Te^R determinant on this plasmid is contained within a 3.55 kb DNA fragment. Insertion mutagenesis with the transposon Tn*1000* was used to obtain tellurite-sensitive and hyposensitive mutants. Four

polypeptides with molecular weights of 41, 23, 22, and 15 kDa were detected using maxicell analysis. The genes have been sequenced and a fifth open reading frame was also detected (Jobling and Ritchie, 1988). Expression of tellurite resistance on this plasmid appears to be inducible by prior exposure to sub-toxic levels of potassium tellurite (Jobling and Ritchie, 1987).

The plasmid MIP233 is the only member of the IncHI3 group of plasmids (Roussel and Chabbert, 1978). It was first isolated in 1972 from *Salmonella ohio*. It is 231 kb in size and encodes resistance to tellurite and phage inhibition as well as the ability to ferment sucrose. This plasmid is thermosensitive for transfer and has a small amount of DNA homology with other H plasmids (Whiteley and Taylor, 1983). However, the short rigid pili produced by this plasmid are serologically distinct from other H pili (Bradley, 1986).

IncHII plasmids. The plasmid pHH1508a was originally isolated from a strain of *Klebsiella aerogenes* obtained from a patient with typhoid fever (Datta et al., 1981). It is 208 kb in size. Besides mediateing resistance to tellurite, pHH1508a determines resistance to streptomycin, spectinomycin, and trimethoprim by means of a Tn7-like transposon (Datta et al., 1981; Bradley et al., 1982; Yan and Taylor, 1987). Like R27, the transfer genes on pHH1508a are arranged in several regions on the plasmid (Yan and Taylor, 1989). A 96 kb deletion mutant of this plasmid was

constructed (pDT1178) which was transfer-deficient but still produced conjugative pili (Yan and Taylor, 1987; Figure 2). This plasmid still expressed tellurite resistance.

Like IncHI2 plasmids which specify tellurite resistance, pHH1508a inhibits the development of some double-stranded DNA bacteriophages including λ, T1, T5, and T7 (Taylor and Summers, 1979; Bradley et al., 1982). This phenotype is called *phi* and appears to involve restriction of the phage DNA (Watanabe et al., 1966; Revel and Georgopoulos, 1969).

Expression of tellurite resistance has also been linked to colicin B resistance on IncHI2, IncHI3 and IncHII plasmids (Maher et al., 1989). Colicin B is bactericidal due to the formation of transmembrane pores in the cytoplasmic membranes of sensitive bacterial cells (Pressler et al., 1986). Bacteria producing colicin B are resistant to its effects by producing immunity proteins (Schramm et al., 1987). The presence of this property has been used as a simple, rapid method of identifying the H plasmids (Rodriguez-Lemoine, 1982).

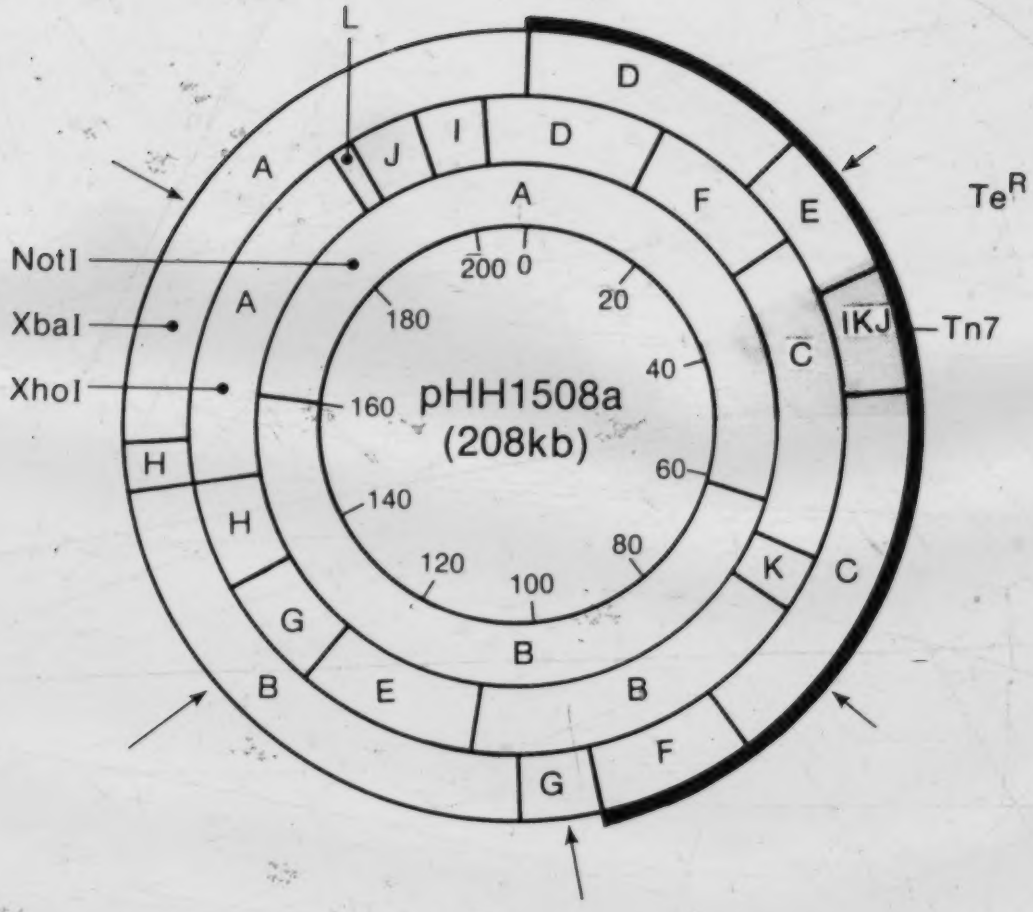


Figure 2. Restriction map of pHH1508a.
Restriction endonuclease sites on the plasmid pHH1508a for *Xba*I, *Xho*I and *Not*I are shown in the outer circles. Map positions in the plasmid are shown in the center in kilobases. Restriction fragments are labelled alphabetically in order of decreasing size. Regions of the plasmid which are required for conjugative transfer are indicated by arrows. The region contained in the deletion mutant, pDT1178, is indicated by the black outer circle. The probable position of the tellurite resistance determinant is indicated by Te^R. (modified from Yan and Taylor, 1987, 1989).

1.5 Objectives of this study.

In this study, the Te^R determinants of the plasmids RK2/RP4 and pHH1508a were cloned and sequenced. The genes involved in tellurite resistance were investigated using the technique of RNA primer extension and the preparation of transcriptional and translational fusions. Tellurite resistance proteins were examined by overexpression in a T7 RNA polymerase/promoter system. Preliminary characterization of the resistance mechanisms encoded by these two plasmids was undertaken.

A comparison of the Te^R determinants from several plasmids will give clues as to how resistance to tellurite evolved. This work will enhance our understanding of the importance of tellurite resistance to bacteria in the environment and in the human body and its role in maintenance of these important antibiotic resistance determinants in pathogenic bacteria.

2. Materials and Methods

2.1 Antibiotics and Media. Unless otherwise stated, antibiotic plates contained the concentrations of antibiotics which are listed in Table 1. All antibiotic plates were made from Luria broth (LB; Gibco) to which 1.5 % agar (Gibco) was added or MacConkey agar (Difco) except tellurite plates which were from brain heart infusion (BHI) agar (Difco) and trimethoprim plates which were from diagnostic sensitivity testing (DST) agar (Oxoid).

Table 1. Antibiotics.

Antibiotic	(Abbreviation)	Concentration in media (µg/ml)
Ampicillin	(Ap)	100
Carbenicillin	(Carb)	500
Chloramphenicol	(Cm)	30
Kanamycin	(Km)	15
Nalidixic acid	(Nal)	24
Potassium tellurite	(Te)	50
Rifampicin	(Rif)	100
Streptomycin	(Sm)	200
Sulfonamide	(Su)	1000
Tetracycline	(Tc)	8
Trimethoprim	(Tp)	10

2.2 Bacterial Strains and Plasmids. The bacterial strains and plasmids used in this study are listed in Tables 2 and 3, respectively. Bacterial strains were stored at -70°C in an autoclaved preserving medium containing 3 % (w/v) trisodium citrate and 40 % glycerol (v/v).

Table 2. Bacterial strains used in this study.

E. coli strain	Relevant characteristics	Reference or source
JM83	Δ*lac-pro*, ϕ80d*lacZ* ΔM15	Vieira and Messing (1982)
JM107	F' *lacI*^{q}*Z* ΔM15	Yanish-Perron et al., (1985)
RG1900	Rif^R	D. E. Taylor
J53-1	Nal^R lac$^+$ *pro met*	Bachman, (1972)
MV12	*recA56*	Thomas et al., (1980)
MV10	*lacY*	Thomas et al., (1980)
JE2571-1	Sm^R Nal^R lac$^+$	Bradley, (1980a)
RG192	Lac$^-$ Rif^R	Taylor and Grant, (1977)
W1485	F'::Tn*1000* lac$^+$	Reed, (1981)
M8820 Mu	*araD139* Δ(*ara-leu*)*7697* Δ(*proAB-argF-lacIPOZYA*) *XIII rpsL*, Mu	Castilho et al., (1984)
PoI1681TR	Mu dI1681 (Km^R) (*ara*::Mucts)3 Δ(*proAB-argF-lacIPOZYA*) *XIII rpsL recA56 srl*::Tn*10* (Tc^R)	Castilho et al., (1984)
poII1681TR	*rpsL*, MudII1681 (Km^R) *ara*::(Mu *cts*)3 *araD*$^-$ leu$^+$ lac$^+$ pro$^+$ *recA56 srl*::Tn*10* (Tc^R)	Castilho et al., (1984)
LE392	F$^-$ *supE44 supF58 hsdR514 galT22 galK2 trpR55 metB1 lacY1 tonA*	Gutierrez et al., (1987)
CC118	Rif Δ*lacX74 phoA*Δ20 *recA1*	Manoil and Beckwith, (1985)
K38	HfrC (λ)	Russel and Model, (1984)
MI1443	Δ(*ampC frdABCD*) *polA*$^+$ str^R *HfrC*	Latour and Weiner, (1987)
DSS106	Δ(*dmsABC*)	D. Sambasivarao[1]
DL16	*recA1*, *ilvE720*::Tn5 (Km^R), *trxA1*	Mark et al., (1977)

[1]Department of Biochemistry, University of Alberta

Table 3. Bacterial plasmids used in this study.

Plasmid	Resistance markers[a]	Source	Reference
pHH1508a (IncHII)	SmSuTpTe *phi*	*Klebsiella aerogenes*	Bradley et al., (1982)
pDT1178	TpTe *phi*	deletion mutant of pHH1508a	Yan and Taylor, (1987)
RK2 (= RP4, RP1) (IncPα)	ApKmTc *fiw*	*Klebsiella aerogenes*, *Pseudomonas aeruginosa*	Ingram et al., (1973); Holloway and Richmond (1973)
RK2Te^R	ApKmTcTe *fiw*	derivative of RK2/RP4	Taylor and Bradley (1987)
pRK2102	Ap	laboratory derived from RK2	Figurski et al., (1982)
pRK2102Te^R	ApTe	laboratory derived from pRK2102	Taylor and Bradley, (1987)
pUC8	Ap	cloning vector	Vieira and Messing, (1982)
pACYC184	CmTc	cloning vector	Chang and Cohen, (1978)
pT7-5	Ap	expression vector	S. Tabor and C. Richardson, (unpublished)
pT7-6	Ap	expression vector	S. Tabor and C. Richardson, (unpublished)

[a] *fiw*, fertility inhibition towards IncW plasmids; *phi*, phage inhibition.

2.3 Plasmid DNA Isolation. The plasmids pHH1508a and pDT1178 were isolated by the sarkosyl lysate method described by Bazaral and Helinski (1968) as modified by Whiteley and Taylor (1983). In this procedure, bacterial strains were grown in rich minimal medium to an OD_{600} of between 0.4 and 0.6. The bacterial cells were treated with a cold sucrose solution (20 % sucrose; 0.1 M NaCl, 0.02 M ethylene diamine tetraacetate (EDTA), pH 8.0) containing 2 mg/ml lysozyme. The cells were lysed with an equal volume of cold sarkosyl lysis buffer (1 % sarkosyl, 0.05 M NaCl, 0.01 M EDTA, pH 8.0) and incubated at room temperature (RT) for 20 minutes (min.). The lysate was treated with 0.5 mg/ml Ribonuclease A at 25°C followed by 0.2 mg/ml Pronase P at 37°C. Cesium chloride (CsCl; Terochem Laboratories, Edmonton, Canada) and ethidium bromide (EtBr) were added and the DNA was subjected to centrifugation in a Beckman preparative ultracentrifuge L8-70 in a Ti-70.1 rotor at 55,000 rpm for approximately 20 hours (h) at 20°C. A second CsCl-EtBr gradient step was carried out for further purification of the plasmid DNA. The plasmid DNA was extracted with iso-amyl alcohol 3 times and ethanol precipitated with 70 % ethanol for 20 min. followed by a precipitation with 95 % ethanol. The pellet was dried under vacuum and resuspended in TE buffer (50 mM Tris, 5 mM EDTA, pH 8.0) or sterile double-distilled water and then stored at 4°C.

All other plasmids were isolated by a scaled-up version of the method of Birnboim and Doly (1979) followed by CsCl-EtBr density gradient centrifugation.

2.4 Restriction Enzyme Digestion. Restriction enzymes (*AccI* (A), *BamHI* (Ba), *BglI* (Bg), *BglII* (Bl), *BssHII* (Bs), *DraI* (D), *EcoRI* (E), *EcoRV* (EV), *HincII* (Hc), *HindIII* (Hd), *HpaI* (Hp), *PstI* (P), *SalI* (Sa), *SmaI* (Sm), *SstI* (Ss), *SstII* (St), *XbaI* (Xb), *XhoI* (Xh)) were obtained from Boehringer Mannheim Biochemicals, Canada, Ltd. (BMC), Montreal, Quebec, or Bethesda Research Laboratories (BRL) Canada, Ltd., Mississauga, Ontario. Restriction enzyme digestions were carried out according to the manufacturers' recommendations using Boehringer Mannheim SuRE/Cut buffers (Appendix 1). Double digests were carried out simultaneously or by performing one digestion, then adding the second enzyme and buffer directly to the digestion mixture and incubating at the second temperature. Restriction digests to be analyzed by electrophoresis were terminated by the addition of 1/10 volume of bromophenol blue (BPB) loading dye (48 % sucrose, 0.25 % bromophenol blue (BioRad), 12 mM EDTA).

2.5 Filling in ends of restriction fragments with the Klenow fragment of DNA Polymerase. The Klenow fragment of DNA polymerase (BMC) was used to fill in the recessed ends of DNA fragments obtained from restriction digests. This method was performed essentially as described by Maniatis et al.,

(1982). Approximately 0.5 µg of the restriction digested DNA was mixed with unlabelled dATP, dCTP, dGTP and dTTP at concentrations of 20 µM each in buffer containing 25 mM Tris-HCl, pH 7.5; 2.5 mM $MgCl_2$; 5 µM β-mercaptoethanol. The mixture was incubated with 0.5 units of Klenow enzyme for 3 h at 15°C.

2.6 Agarose Gel Electrophoresis. Samples of restriction endonuclease digests were mixed with BPB loading dye and subjected to electrophoresis at 50-100 V in horizontal gels composed of 0.6 to 1.0 % agarose in Tris-borate-EDTA buffer (0.09 M Tris, 0.09 M boric acid, 2.0 mM EDTA, pH 8.3) (Portnoy et al., 1981). Gels were stained in a dilute EtBr solution (~ 5 µg/ml). DNA fragments were visualized by exposure of the gel to ultraviolet (UV) light. The sizes of restriction fragments were determined with reference to bacteriophage lambda DNA digested with *Hind*III (Davis et al., 1980). Restriction mapping was carried out by comparing the results of double and single digests.

2.7 Extraction of DNA fragments from low melting point agarose. Restriction endonuclease digests were subjected to electrophoresis as described above except that low-melting-point agarose (BRL) was used. The gels were stained with EtBr and the DNA fragments were excised as quickly as possible to minimize exposure to the UV light. The DNA was extracted from the low-melting-point agarose by suspending in

100 µl TE and heating to 65°C for 10 min. An equal volume of Tris-equilibrated phenol was added and the mixture was vortexed and subjected to centrifugation for 3 min. in an Eppendorf microfuge at 12,000 rpm. The aqueous phase (top) was collected, mixed with an equal volume of butanol and subjected to centrifugation. The aqueous phase (bottom) was then mixed with an equal volume of chloroform and subjected to centrifugation. Finally the aqueous phase (top) was removed, 1/10 volume of 3 M NaOAc (pH 4.8) was added and the DNA was precipitated with ethanol.

2.8 Expression of tellurite resistance by RP4 and pHH1508a. Cultures of RP4 and pHH1508a were grown overnight in LB. Side arm flasks containing 25 ml of fresh LB were inoculated with 0.1 ml of overnight culture. Induction was performed by adding potassium tellurite to some of the flasks to give a final concentration of 0.1 or 0.26 µg/ml (~ 1 mM) and cultures were allowed to grow for a short period of time at 37°C with shaking. Cultures were challenged by adding a high concentration of potassium tellurite (19.5 µg/ml) and incubation at 37°C was continued. Growth rates were followed by measuring the optical density at 600 nm in a Coleman Junior IIA spectrophotometer.

2.9 Cloning of Te^R determinants. Te^R clones were constructed using methods described by Maniatis et al., (1982). Restriction enzyme digested DNA was ligated into the multiple

cloning site of pUC8 (Vieira and Messing, 1982) using bacteriophage T4 DNA ligase (BRL). Recombinant DNA was transformed into *E. coli* JM83 (*lacZ* ΔM15) using the calcium chloride method of Cohen et al., (1972). In this method, 40 ml of LB was inoculated with 1 ml of an overnight culture of the *E. coli* recipient and incubated at 37°C (or 30°C for temperature-sensitive strains) with shaking for exactly 2 h. The culture was subjected to centrifugation for 1 min. at 7000 rpm using a Beckman JA-21 rotor. The pellet was suspended in 20 ml of cold 0.03 M $CaCl_2$ and incubated on ice for 20 min. The suspension was subjected to centrifugation at 7000 rpm for 30 seconds and the pellet was resuspended in 2 ml of 0.03 M $CaCl_2$. The DNA to be transformed was mixed with 200 µl of cell suspension and incubated for 30 min. on ice. Cells were subjected to a heat shock for 1 min. and 45 seconds at 42°C (or 30°C in some experiments). An equal volume of LB (200 µl) was added and the cells were incubated at either 37°C or 30°C for 30 to 60 min. and aliquots were plated on antibiotic selection plates. pUC8 transformants were selected on LB agar plates containing either Ap_{100} or $Carb_{500}$, as well as XGal (BRL) and IPTG (Sigma) or on MacConkey plates containing either Ap_{100} or $Carb_{500}$ (Vierra and Messing, 1982). Lactose-negative colonies were then tested for Te^R. Further subclones were constructed by cloning of Te^R regions into restriction enzyme sites in the plasmid pACYC184 (Chang and Cohen, 1978) followed by recloning into pUC8.

2.10 Dot Blot Hybridization. DNA fragments were isolated from low-melting-point agarose and radioactively labelled by nick translation as described by Rigby et al., (1979). In this method, the DNA was mixed with dCTP, dGTP, and dTTP at concentrations of 20 µM each in a buffer of 25 mM TrisHCl, pH 7.5, 2.5 mM $MgCl_2$, and 5 µM β-mercaptoethanol. Approximately 50 µCi of [α-^{32}P]dATP was added. The reaction mixture was preincubated with about 2 x 10^{-7} µg of DNAse at 15°C for 15-30 min. Approximately 0.5 units of DNA Polymerase I was added and the incubation was continued for 2-4 hr at 15°C. The radioactively labelled DNA was precipitated with ethanol after the addition of 10-20 µg yeast tRNA and 1/10 volume of 3 M NaOAc (pH 4.8).

Dot blots were prepared as described previously (Kafatos et al., 1979). DNA concentrations were determined by measurement of the absorbance at 260 nm in a spectrophotometer. DNA was denatured in 0.3 M NaOH at RT for 15 min. and serial five-fold dilutions were prepared in 1 M ammonium acetate. The nitrocellulose filter was soaked in 1 M ammonium acetate for 20 min. 50 µl volumes of diluted DNA samples were applied to the nitrocellulose filter using a dot blot apparatus. The filter was air dried at RT and baked at 65°C for 16 h.

DNA-DNA hybridization was performed as described by Portnoy et al., (1981). The filter was incubated with pre-hybridization solution (50 % formamide; 0.75 M sodium

chloride; 85 mM sodium citrate; 0.1 % sodium dodecyl sulfate (SDS); 1 mM EDTA; 0.2 % ficoll 400; 0.2 % bovine serum albumin (BSA); 0.2 % polyvinyl pyrrolidone) in a heat-sealed plastic bag for 3 h at 37°C. The prehybridization solution was drained out of the bag and replaced with 2.5 ml of prehybridization solution. The radioactive DNA probe was denatured by boiling for 10 min. with 625 µg sonicated herring sperm DNA. The radioactive mixture was added to the filter and prehybridization solution in the plastic bag and incubation was continued at 37°C for 16 h. The filter was removed and rinsed with wash buffer (75 M sodium chloride; 85 mM sodium citrate; 0.1 % SDS; 1 mM EDTA, pH 7.0) twice at RT. This was followed by two incubations in wash buffer at 65°C for 45 min. each. The filter was then washed twice with 2 X SSC (0.3 M sodium chloride; 34 mM sodium citrate) at RT. The filter was then air dried and exposed to Kodak X-ray film (X-Omat AR) for various periods of time at -70°C.

2.11 Isolation of tellurite-sensitive Tn*1000* insertion mutants. Plasmids carrying the Te^R genes of RK2 or pHH1508a were transformed into *E. coli* K12 W1485. This strain contains the F plasmid (which carries the transposon Tn*1000* ($\gamma\delta$)) integrated into its chromosome. The resulting strains, selected on ampicillin plates, were mated with *E. coli* JE2571-1 (Nal^R) overnight in Penassay broth (Difco) at 37°C. The mating mixture was diluted in phosphate buffer and transconjugants were selected on $Nal_{24}Carb_{500}$ media. *E. coli*

JE2571-1 strains into which the clones had been transferred during conjugative transfer of the F factor were tested for loss of resistance to tellurite due to insertion of Tn*1000* by plating on media containing 50 μg/ml of potassium tellurite.

2.12 Analysis of Tn*1000* insertions. Tn*1000* (γδ) is about 5.7 kb in length (Guyer, 1978). An *Sst*I site is located close to the δ end (Figure 8). Measuring from the γ end, there are *Xho*I sites at 1.4 kb and 4.6 kb, a *Hinc*II site at 2.8 kb, a *Bam*HI site at 5.3 kb, and a *Sma*I site at 3.2 kb (Guyer, 1978). The position and orientation of the Tn*1000* insertions into the Te^R clone pDT1364 were determined by comparing *Hinc*II digestions, double digestions with *Sma*I and *Sst*I, and double digestions with *Xho*I and *Sst*I with the restriction maps of pDT1364 and Tn*1000*. Similarly, the sites of insertion of Tn*1000* into the Te^R clone pDT1558 were determined from single and double digestions using the enzymes *Bam*HI, *Sma*I, *Xho*I, and *Sst*I. The positions of some Tn*1000* insertions were determined by subcloning the junction points into M13mp18 or M13mp19 followed by single-stranded DNA sequencing, or by sequencing plasmids containing Tn*1000* insertions directly using oligonucleotide primers complementary to the γ or δ ends of the transposon.

2.13 MIC Determination. Determination of the minimum inhibitory concentration (MIC) of potassium tellurite for Te^R

bacteria and Te^S derivatives was performed using the agar dilution method (Washington, 1985). Brain heart infusion agar was prepared containing two-fold dilutions of potassium tellurite from 0.125 to 512 µg/ml. Bacterial strains to be tested were incubated in LB for 5 h at 37°C with shaking, then diluted 1 in 20 in Penassay broth. A Steer's replicator was used to inoculate equal volumes of each strain on the agar dilution plates. The lowest concentration of tellurite which inhibited growth was determined to be the MIC.

Determination of MICs by the broth dilution method was performed as described above except that bacteria were inoculated into a series of tubes of LB containing two-fold dilutions of potassium tellurite (Jones et al., 1985). Determination of MICs under anaerobic conditions was performed by incubating plates or broth cultures in anaerobic jars.

2.14 Fertility inhibition properties of IncPα Te^R plasmids. Plasmids carrying various parts of the Te^R region of RK2 wer transformed into *E. coli* RG1900 (Rif^R) carrying the IncW plasmid R388. Both plasmids were maintained in the resulting strain by selection on plates containing both trimethoprim and ampicillin. Equal volumes of 8 h cultures of donor and recipient (*E. coli* JE2571-1 (Nal^R)) were mixed and incubated for 1 h on a LB plate at 37°C. Dilutions were made in phosphate buffer and 100 µl aliquots were plated on Nal Tp plates to determine the number of transconjugants and on Rif

plates to determine the number of donors. The mating frequency of R388 from each strain was determined by dividing the number of transconjugants by the number of donors.

2.15 Phage inhibition properties of IncHII Te^R plasmids. Bacteriophage lambda was prepared as described previously (Maniatis et al., 1982). Dilutions were made in lambda diluent (10 mM Tris-HCl, pH 7.5; 100 mM NaCl; 10 mM $MgSO_4$; 2 % gelatin (w/v)), mixed with soft LB agar and an aliquot of an overnight culture of the strain of *E. coli* to be tested, and poured on an L agar plate containing 2 mM $MgCl_2$. The plaques were counted after O/N incubation at 37°C.

2.16 Analysis of proteins by polyacrylamide gel electrophoresis. Proteins to be analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were dissolved in sample buffer (65 mM Tris-HCl, pH 6.8; 10 % glycerol; 2 % SDS; 0.05 % β-mercaptoethanol; 0.05 % BPB) and boiled for 5 min. Polypeptides were separated on 10-15 % polyacrylamide gels containing 0.1 % SDS using the method described by Laemmli (1970). Gels were fixed in 40 % methanol, 10 % glacial acetic acid and stained with 0.2 % Coomassie Brilliant Blue R250.

Nondenaturing polyacrylamide gels were prepared as above except that SDS was omitted in all solutions. The sample was suspended in a solution of 10 % running buffer and 10 % sucrose, and was not boiled before loading. The gel was

subjected to electrophoresis at 4°C. The gel was dried briefly on filter paper and soaked in a solution of 50 mM 3-(N-Morpholino)propanesulfonic acid (MOPS), 1 mM NADH for 5 min. followed by the addition of K_2TeO_3 to a final concentration of 1 mM.

2.17 Analysis of plasmid proteins by *in vitro* transcription-translation. Plasmid-encoded proteins were expressed using an *E. coli in vitro* transcription-translation system (Amersham Canada, Oakville, Ontario, Canada). Polypeptides synthesized in this system were labelled with L-[^{35}S]-methionine (New England Nuclear Corp, Boston, Mass.) and separated by SDS-PAGE as described above. In some experiments, the gel was prepared for fluorography by soaking in dimethyl sulfoxide (DMSO) followed by a solution of the fluor diphenyl oxazole (PPO) in DMSO (Bonner and Laskey 1974). The gel was washed with water, dried under vacuum and then exposed to X-ray film for various times at -70°C. The sizes of the polypeptides were determined by comparison to ^{14}C-methylated standard molecular weight proteins obtained from Amersham Canada. The proteins and their molecular weights are as follows: myosin, 200,000; phosphorylase-b, 92,500; bovine serum albumin, 69,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; lysozyme, 14,400.

2.18 Preparation of *lac* fusions with mini-Mu. Plasmids containing the Te^R regions of RK2Te^R or pHH1508a inserted into

pACYC184 were transformed into the mini-Mu strains PoI1681TR and PoII1681TR as described by Cohen et al., (1972), except that the heat pulse was at 30°C for 2 min. rather than at 42°C. In addition to mini-MudI or mini-MudII, these strains contain a complementing Mu cts prophage in the chromosome so that transposition and phage growth can be induced by heating to 42°C (Castilho et al., 1984).

Transducing lysates of these strains were prepared as described by Castilho et al., (1984). The mini-Mu strains PoI1681TR and PoII1681TR containing the Te^R derivatives of pACYC184 were grown O/N at 30°C in LB containing Cm and Km. Cultures were diluted 1/100 in LB and incubated at 30°C with shaking until the OD_{600} was about 0.2. The temperature was raised to 42°C and the cultures were incubated until lysis occurred. A few drops of chloroform were added to the culture and incubation with shaking was continued for 10 min. $MgSO_4$ and $CaCl_2$ were added to final concentrations of 2 mM and 0.2 mM respectively. The culture was subjected to centrifugation and the supernatant was saved.

This transducing lysate was used to infect *E. coli* M8820Mu as follows: *E. coli* M8820Mu was grown O/N at 37°C. The culture was diluted 1/100 in LB containing 2 mM $MgSO_4$ and 0.2 mM $CaCl_2$ (LCM) and this culture was allowed to grow at 37°C with shaking for 4 h. Equal volumes of phage lysate and M8820Mu culture were mixed. The mixture was incubated at 30°C for 30 min. without shaking. The mixture was then centrifuged and resuspended in 0.5 ml LCM. Aliquots were

spread on MacConkey plates containing Cm to select for the drug resistance marker on pACYC184 and plates were incubated at 30°C for 48 h. Colonies expressing the Lac phenotype were tested for resistance to kanamycin (16 µg/ml), the marker present on both the mini-MuPoI (transcriptional fusion) and mini-MuPoII (translational fusion) phages, as well as for resistance to potassium tellurite (50 µg/ml).

A series of strains containing mini-MuPoI and mini-MuPoII insertions, which were unable to grow on 50 µg potassium tellurite per ml, were thus identified. Positions of mini-Mu insertions were identified by analysis with the restriction endonucleases *Bam*HI, *Dra*I, *Eco*RI, *Hinc*II, *Sma*I, and *Xho*I.

2.19 Preparation of alkaline phosphatase fusions using Tn*phoA*. A high titre λTn*phoA* bacteriophage stock was prepared as follows: λTn*phoA* diluted in λ diluent (10 mM Tris, pH 7.4; 10 mM $MgSO_4$) was incubated with 100 µl of an overnight culture of *E. coli* LE392 in LBM (LB plus B1, 0.01 M $MgSO_4$, and 0.2 % maltose) for 15 min. at RT; the mixture was inoculated into 3 ml of top agar (1 % tryptone; 0.5 % NaCl; 0.65 % Noble agar [Difco]) and poured onto LB agar plates; after O/N incubation at 37°C, 1-2 ml of λ diluent and 1 ml of chloroform were added to the plate and the liquid and top agar were transferred to a sterile glass Corex tube and subjected to centrifugation. The supernatant was stored at 4°C and the phage titre determined.

Tn*phoA* insertions into pDT1558 and pDT1364 were prepared using λTn*phoA* as described by Gutierrez et al., (1987). The target plasmids were transformed into *E. coli* CC118. The resulting strains were incubated with λTn*phoA* at 3-4 plaque forming units per cell, then plated on media containing Km and Ap. Expression of alkaline phosphatase was detected by the presence of blue colonies on LB agar containing 5-bromo-4-chloro-3-indolyl-phosphate (XP; Sigma). The mixture of bacteria containing insertions in either the chromosome or the plasmid was scraped off the plate and plasmid DNA was isolated by the method of Birnboim and Doly (1979). The resulting plasmid DNA mixture was transformed back into CC118 and transformants with Tn*phoA* insertions in the plasmid were selected on media containing Ap, Km and XP. Blue colonies were streaked out and tested for tellurite resistance and the site of insertion of Tn*phoA* into the plasmid was determined. Tn*phoA* mutagenesis experiments on each target plasmid were performed at least 3 times to ensure the isolation of several independent insertion events.

2.20 <u>DNA Sequencing.</u> DNA sequencing reactions were performed using the Sequenase kit obtained from United States Biochemicals (Cleveland, Ohio). [α-^{35}S]dATP was obtained from New England Nuclear. Single-stranded DNA sequencing by the chain-termination method (Sanger et al., 1977) was carried out using the bacteriophages M13mp18 and M13mp19 (Yanisch-Perron et al., 1985). For double-stranded

sequencing, the DNA was mixed with primer and chemically denatured in a solution of 0.2 M NaOH for five min., then neutralized with 0.2 volumes of 3 M NaOAc (pH 4.8) and then precipitated twice with ethanol (Hattori and Sakaki, 1986). A second amount of primer was added and hybridization was carried out at 37°C for 15 min.. Oligonucleotide primers were kindly synthesized by Dr. Ken Roy at the University of Alberta. Primers were resuspended in water at a concentration of 5-10 µg/ml, as determined by the absorbance at 260 nm (Sambrook et al., 1989). Sequencing reactions were subjected to electrophoresis in 6 % acrylamide gels for 2-10 h at a constant power to maintain a temperature of 50°C using the BioRad Sequencing apparatus. Analysis of the nucleotide sequences and derived protein sequences was carried out on an IBM PC-XT computer using the Beckman Microgenie program Version 6 (Queen and Korn, 1984) or on a Macintosh Plus or SE computer using the DNA Strider program (Marck, 1988).

2.21 Isolation of RNA. RNA to be used for primer extension was isolated using a method based on Aiba et al., (1981). One ml of fresh bacterial culture carrying the appropriate Te^R or Te^S plasmid was centrifuged and dissolved in 300 ml RNA buffer (10 mM Tris, 100 mM NaOAc, 5 mM $MgCl_2$, pH 5.3). The suspension was placed at 65°C and 30 µl of 10 % SDS was added. Immediately, an equal volume of 65°C phenol equilibrated with RNA buffer was added. The mixture was shaken and another volume of chloroform was added. After

centrifugation, the aqueous phase was extracted with phenol/chloroform 2 or 3 more times, then ethanol precipitated. The RNA was dried and resuspended in 200 µl sterile double-distilled water. Presence of RNA was confirmed by electrophoresis of a small aliquot through a 1 % agarose gel made in 0.01 M Na_2HPO_4 buffer, pH 7.0. The concentration of RNA was determined to be approximately 0.5 mg/ml by measuring the absorbance at 260 nm (Sambrook et al., 1989).

2.22 RNA Primer Extension. Primer extension was carried out as follows: 6 µl of RNA (~ 3 µg) was mixed with 2 µl of primer (~ 10-20 ng) and 2 µl of 5 x concentrated reverse transcriptase buffer (0.25 M Tris HCl, pH 8.3; 0.2 M KCl; 35 mM $MgCl_2$). The mixture was incubated for 2 min. at 65°C and then allowed to cool to RT over about 30 min. The deoxy nucleotides dCTP, dGTP, and dTTP were added to give final concentrations of 100 µM of each and dithiothreitol was added to a concentration of 5 mM. Approximately 5 µl (50 µCi) of [α-^{35}S]dATP was added and the mixture was incubated with 2-3 units of AMV Reverse Transcriptase (ProMega) for 2 min. at 42°C. Non-radioactive dATP was added to a concentration of 100 µM and the incubation was continued for another 20 min. at 42°C. An equal volume of stop solution (United States Biochemicals) was added and the reaction mixture was boiled for 2 min. before loading approximately 4 µl on a sequencing gel.

2.23 Expression of proteins using the T7 RNA polymerase/promoter expression system. The protocol for labelling of plasmid proteins using the T7 RNA polymerase/promoter system was kindly provided by Stan Tabor (Harvard Medical School). Parts of the Te^R regions of RK2 and pHH1508a were cloned into pT7-5 or pT7-6 (Ap^R; S. Tabor and C. Richardson, unpublished). The resulting plasmids were transformed into *E. coli* K38 (Russel and Model, 1984) containing the plasmid pGP1-2 which carries the gene for T7 RNA polymerase under the control of the inducible λ P_L promoter (Km^R; Tabor and Richardson, 1985). The resulting strains were grown overnight in LB with Ap and Km at 30°C. The cultures were diluted 1 in 20 in LB plus antibiotics and incubated at 30°C with shaking for approximately 4 h or until the OD_{590} reached approximately 1.5. The temperature was raised to 42°C for 30 to 45 min. and then reduced to 30°C for another 2 h. The cells were harvested and resuspended in SDS-PAGE loading buffer for application to SDS-PAGE.

Proteins expressed using the T7 system were labelled with L-[^{35}S]-methionine as follows: Overnight cultures of cells carrying both pGP1-2 and the T7 recombinant plasmid were diluted 1 in 20 in LB and incubated for 1.5 h at 30°C with shaking. 400 µl of culture was removed and subjected to centrifugation in an eppendorf microfuge. The cell pellet was washed and resuspended in 1 ml of M9 media supplemented with 1 % Difco methionine assay medium and 1 % glucose.

After incubation at 30°C for 60 min., the temperature was raised to 42°C for 15 min. Rifampicin, an inhibitor of *E. coli* RNA polymerase, was added to a final concentration of 200 µg/ml and the cells were incubated at 42°C for another 10 min. The temperature was reduced to 30°C for 20 min. and 2 µl (10 µCi) [^{35}S]met was added followed by another 60 min. incubation at 30°C. The cells were collected by centrifugation and resuspended in 50-100 µl SDS loading buffer.

2.24 Electron microscopy. Te^R bacteria were grown on agar or in broth in the presence of 50 µg/ml potassium tellurite. Te^S *E. coli* (pUC8) was grown O/N in LB. Potassium tellurite was then added to a final concentration of 100 µg/ml, and cells were incubated for 2-3 h until a black color was observed. Whole unstained unfixed bacteria were directly examined in a Phillips 300 transmission electron microscope. Bacteria were fixed in 2.5 % glutaraldehyde in cacodylate buffer and ultrathin sections were prepared as described previously (Taylor et al., 1988). Thin sections were stained with 5 % uranyl acetate in methanol. Electron microscopy was performed by Richard Sherburne and Ben Bablitz.

2.25 Detoxification of tellurite media by Te^R bacteria. Media-conditioning experiments on agar were performed as described by Andremont et al., (1986). The tellurite-sensitive test strain used was *E. coli* JM83. In agar-

conditioning experiments, JM83 was scraped from an LB plate and resuspended in broth to the density of a 0.5 McFarlane standard, then streaked onto a BHI plate using a sterile swab. Tellurite disks were prepared by inoculating them with 20 µl of various dilutions of potassium tellurite. The disk was placed in the center of the plate and Te^R and Te^S test strains of *E. coli* were streaked outwards from the disk to the edge of the plate. The plates were then incubated at 37°C for approximately 24 hr.

Media-conditioning experiments in broth were performed as follows: Te^R and Te^S test strains of bacteria were inoculated into BHI broth with or without 64 µg/ml of tellurite and incubated overnight at 37°C with shaking. The cultures were centrifuged and the supernatants were filter-sterilized. Serial dilutions of the sterile filtrate were prepared in 2 ml volumes of BHI broth and inoculated with 12.5 µl of an overnight culture of *E. coli* JM83 which had been diluted to the density of a 0.5 McFarlane standard. The tubes were incubated at 37°C overnight with shaking and the presence or absence of growth recorded.

2.26. <u>Assays for tellurite reductase activity in whole cells</u>. The reduction of tellurite to black tellurium by whole cells carrying Te^R and Te^S plasmids was measured using a Klett-Summerson colorimeter with green filter no. 50. Cultures were grown overnight at 37°C in LB containing 100 µg/ml ampicillin. Potassium tellurite was then added to a final

concentration of 100 µg/ml. At various times after the addition of tellurite, 1 ml aliquots were removed, diluted 1/10 in water and the Klett reading measured.

The final concentrations of inhibitors added were: sodium azide, 100 mM, potassium cyanide, 50 mM, and 2,4-dinitrophenol, 2.5 mM. Carbon substrate buffers contained glucose, succinate, D(-)lactate, or glycerol at a concentration of 0.01 M in a buffer of 22 mM KH_2PO_4, 40 mM K_2HPO_4, 1.7 mM sodium citrate, 0.4 mM $MgSO_4$, 7.5 mM $(NH_4)_2SO_4$, pH 7.0.

In experiments to test the use of tellurite as an electron acceptor under anaerobic conditions, bacteria were grown with stirring at 37°C in anaerobic Klett flasks in mimimal media containing 10 mM glycerol, 100 mM potassium phosphate, pH 6.8, 15 mM ammonium sulfate, 0.05 % Casamino Acids, 0.003 % proline, and metal ions [400 mM $MgSO_4$; 25 mM $MnSO_4 \cdot 2H_2O$; 1 mM $Fe_2(SO_4)_3 \cdot nH_2O$; 5 mM $CaCl_2$], (Bilous and Weiner, 1985) In addition, fumarate (final concentration 40 mM), DMSO (final concentration 70 mM) and/or tellurite (final concentration 0.4-4 mM) were added as potential electron acceptors.

3. Results

3.1 Constitutive expression of tellurite resistance by RP4 and pHH1508a.

The expression of Te^R by the IncHI2 plasmid pMER610 appears to be inducible by tellurite (Jobling and Ritchie, 1987). Addition of tellurite to exponentially growing cultures of *E. coli* (pMER610) caused a lag in growth as measured by the optical density of the culture at 600 nm. This slowing of the growth rate could be abolished by the prior addition of sub-toxic levels of tellurite.

The expression of Te^R by the IncPα plasmid RP4 (Figure 1) and the IncHII plasmid pHH1508a (Figure 2) was compared using the same method. Early log phase bacterial cultures of either *E. coli* (RP4) or *E. coli* (pHH1508a) were challenged with high concentrations of potassium tellurite (19.6 µg/ml; ~78 µM). No difference was seen in the growth rates of those cultures which had been previously exposed to low concentrations of tellurite (0.26 µg/ml; ~1 µM) compared to those cultures which were not previously exposed (Figure 3). This suggests that the Te^R determinants of RP4 and pHH1508a are expressed constitutively in *E. coli*.

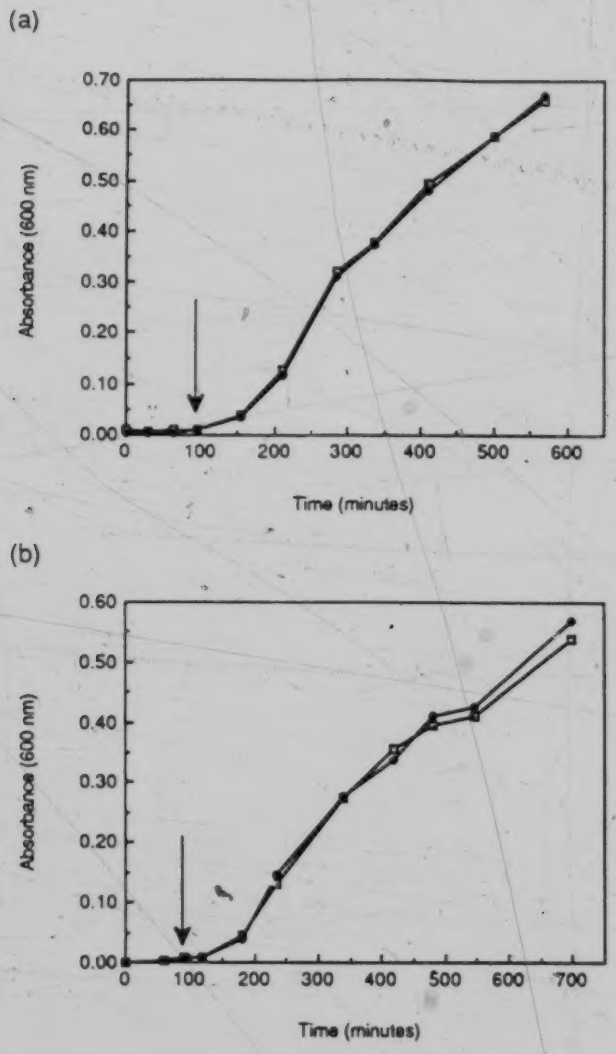


Figure 3. Expression of Te^R by RP4 and pHH1508a.
Overnight cultures of RP4 (a) and pHH1508a (b) were diluted in fresh Luria broth with (-◆-) or without (-□-) subinhibitory concentrations of potassium tellurite (0.26 µg/ml) and cultures were incubated for a short time at 37°C with shaking. Following challenge with a high concentration of tellurite (19.5 µg/ml; indicated by arrow) growth rates were followed by measuring the optical density at 600 nm.

3.2 Cloning of the tellurite resistance determinants.

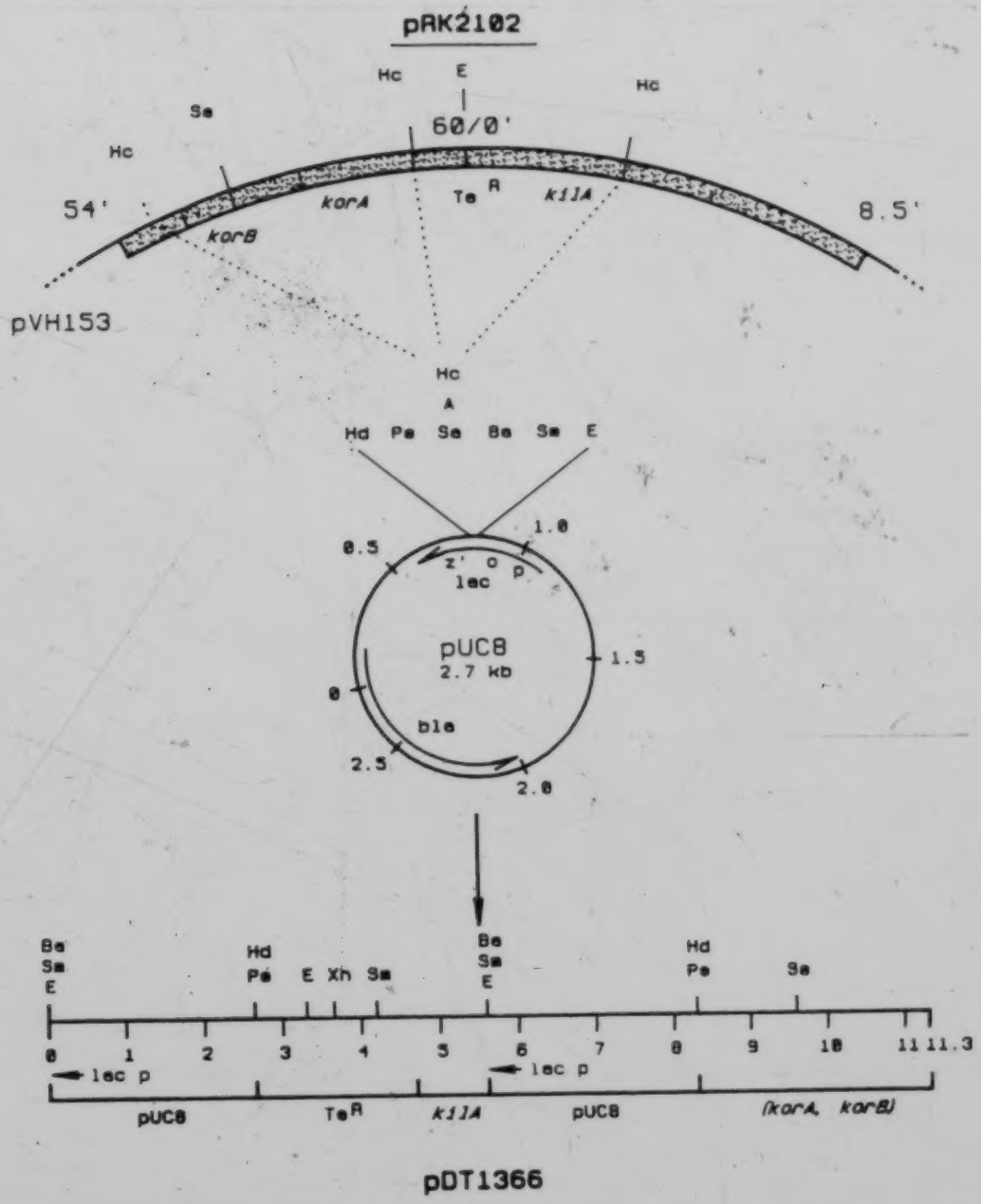
The plasmid pRK2102 carries a large DNA fragment corresponding to the region between 54'-60/0'-8.5' kb on the map of RK2 (RP4) in the vector pVH153 (Figure 1; Figure 4) (Figurski et al., 1982). A variant of this plasmid which expressed resistance to tellurite was isolated by D. E. Bradley (Taylor and Bradley, 1987).

Using the restriction enzyme *Hinc*II, the Te^R region from pRK2102 was cloned into the *Hinc*II site within the multiple cloning region of pUC8 creating the $Te^RAp^Rlac^-$ plasmid pDT1366 (Figure 4). pDT1366 carries two pUC8 sequences separated by two *Hinc*II fragments of RK2 (Figure 5a; Walter and Taylor, 1989). Since the enzyme *Hinc*II creates blunt ends, these sites were lost during re-ligation, and no *Hinc*II sites are present in this plasmid. According to Young et al., (1985), the *Hinc*II site approximately 2.2 kb away from the *Eco*RI site in RK2 is in the promoter of the *kilA* gene which is transcribed in the leftward direction on the map of RK2. Because of the orientation of the second pUC8 sequence in pDT1366, it is possible that the *kilA* gene is being expressed from a hybrid promoter formed during ligation of the fragments or from the β-galactosidase promoter of pUC8 (Figure 4).

The *korA* and *korB* genes are located within the *Hinc*II fragment between 56.5 kb and 59.6 kb on the map of RK2 (Bechhofer et al., 1986). pDT1366 appears to contain this

Figure 4. Cloning of the IncPα Te^R determinant.

Circular maps of the plasmids used in cloning of the Te^R determinant of RK2 are shown (not to scale). The plasmid pRK2102 (top) carries the region from 54'-60/0'-8.5' (kb) on the map of RK2 (shaded) in the vector pVH153. The Te^R plasmid pDT1366 was constructed by shot-gun cloning of *Hinc*II restriction fragments from pRK2102 into the *Hinc*II site within the multiple cloning region of pUC8. The resultant plasmid contains two separate *Hinc*II fragments from pRK2102, one carrying the Te^R and *kilA* genes, and a second which appears to carry the *korA* and *korB* genes. The direction of transcription from the *lac* promoter (lac p) is indicated in pUC8 and pDT1366.


pRK2102
Hc
E
Hc
Sa
60/0'
Hc
54'
korA
Te R
klIA
8.5'
korB
pVH153
Hc
A
Hd Pa Sa Ba Sa E
1.0
0.5
z' o p
lac
pUC8
2.7 kb
1.5
0
bla
2.5
2.0
Ba Sa E
Hd Pa
E Xh Sa
Ba Sa E
Hd Pa
Sa
0 1 2 3 4 5 6 7 8 9 10 11 11.3
lac p
lac p
pUC8
TeR
klIA
pUC8
(korA, korB)

pDT1366

*Hinc*II fragment and therefore probably encodes both *korA* and *korB*. These genes should prevent the *kilA* gene from killing the host cell.

The $Te^{R}Cm^{R}Tc^{S}$ subclone pDT1555 (Figure 5b) was constructed by cloning the *Bam*HI-*Hin*dIII fragment of pDT1366 containing the Te^{R} region and the *kilA* gene of RK2 into the corresponding sites in pACYC184 (Chang and Cohen, 1978), thus eliminating expression of tetracycline resistance by this plasmid. This was followed by recloning of this region into the *Hinc*II site of pUC8 using the Klenow fragment of DNA polymerase to create blunt ends on the insert (Figure 5c). Since the pUC8 sequence in the resulting plasmid, pDT1558, is in the opposite orientation (relative to the Te^{R} insert) from those in pDT1366, it was thought that *kilA* might not be expressed by this plasmid. The initial isolation of pDT1366 suggested that the *korA* and *korB* genes may play a role in the expression of Te^{R}. The absence of the *korA* and *korB* genes on pDT1558 indicates that these genes are not required for expression of this Te^{R} determinant.

The plasmid pDT1178 is a deletion derivative of pHH1508a (Yan and Taylor, 1987). It is approximately half the size but still expresses resistance to tellurite (Figure 2). The Te^{R} region of pDT1178 was cloned into the *Sal*I site of pUC8 creating the $Te^{R}Ap^{R}lac^{-}$ plasmid pDT1364 (Figure 6a; Walter and Taylor, 1989). This plasmid carries a 7 kb *Sal*I fragment of pDT1178. The plasmid pDT1556 (not shown) was constructed by ligating a *Sal*I fragment of pHH1508a into pUC8 and

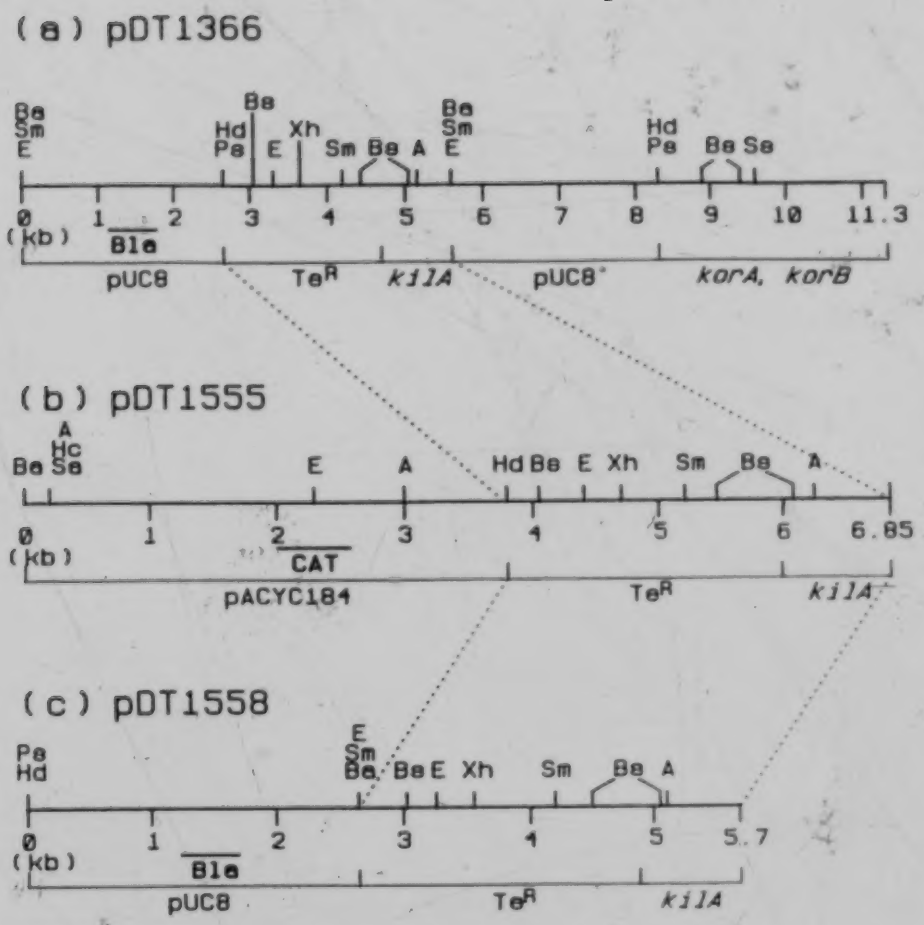


Figure 5. Restriction map of IncPα Te^R subclones.
(a) pDT1366 was obtained by ligating *Hinc*II fragments of the RK2 derivative pRK2102 to *Hinc*II digested pUC8 and selecting for tellurite resistance. (b) pDT1555 was derived by cloning the *Bam*HI-*Hin*dIII fragment of pDT1366 carrying the Te^R and *kilA* genes of RK2 into the corresponding sites in pACYC184. (c) pDT1558 was constructed by subcloning the *Bam*HI-*Hin*dIII fragment from pDT1555 carrying the Te^R and *kilA* genes of RK2 back into pUC8.

selecting for a Te^R lac^- phenotype. The restriction map of this plasmid is identical to that of pDT1364 except that the orientation of the insert with respect to pUC8 is reversed. Since Te^R is expressed in both orientations, this suggests that the Te^R genes have been cloned with their own promoter.

The exact location of the Te^R determinant on the map of pHH1508a is currently under investigation. After initial isolation of pDT1364, the possibility existed that the insert DNA carrying Te^R was derived from the fusion point of pHH1508a *Xba*I fragments D and F in pDT1178 (Figure 2). Thus it could contain sequences from opposite sides of pHH1508a, making the localization of Te^R on this plasmid more difficult. However, since the same fragment was cloned from both pDT1178 and pHH1508a, it appears that the insert of pDT1364 was not derived from this region. The plasmid pDT1364 has sites for the restriction enzymes *Xba*I and *Xho*I located fairly close together on the insert DNA. Therefore, the insert appears to be derived from the region near Tn7 on pDT1178 (Figure 2) since this is the only region on this plasmid where *Xba*I and *Xho*I sites are found this close together (Yan and Taylor, 1987, 1989).

The Te^R plasmid pDT1557 (Figure 6b) was constructed by inserting the *Sal*I fragment of pDT1364 into the *Sal*I site of pACYC184, eliminating expression of the tetracycline resistance determinant. The restriction map of the insert DNA in pDT1364 and pDT1557 shows no similarity to that of pDT1558 or to the Te^R determinant of pMER610 studied by

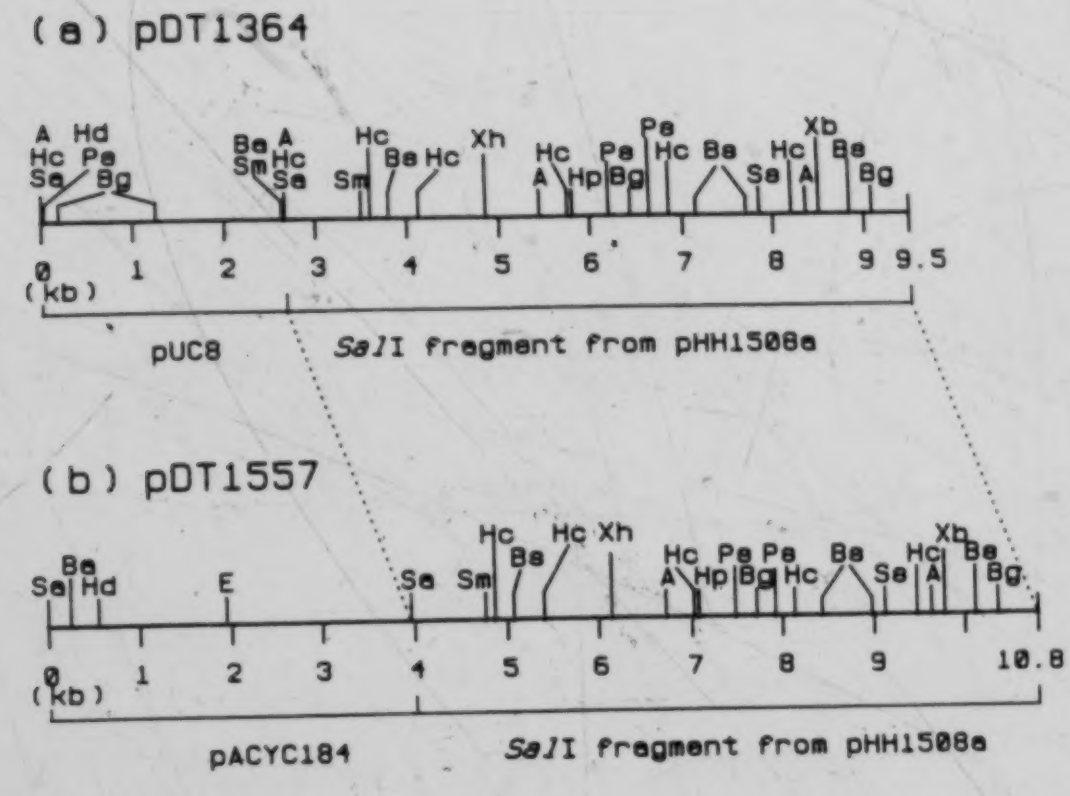


Figure 6. Restriction map of IncHII Te^R subclones.
(a) pDT1364 was obtained by cloning the Te^R region of the IncHII plasmid pHH1508a (Figure 2) into the *Sal*I site of pUC8. (b) pDT1557 was constructed by cloning the *Sal*I fragment of pDT1364 carrying the Te^R determinant from pHH1508a into the *Sal*I site of pACYC184.

Jobling and Ritchie (1987), suggesting that the three determinants are not closely related.

3.3 Lack of homology between Te^R determinants by dot blot hybridization.

The technique of DNA-DNA hybridization was used to determine if there was any homology between the Te^R determinants of RK2Te^R and pHH1508a. Four-fold dilutions were made from preparations containing equal amounts of DNA fragments specifying Te^R from RK2 and pHH1508a. The DNA was denatured, applied to nitrocellulose, and hybridized with radioactively-labelled Te^R determinant from pHH1508a (Figure 7; Walter and Taylor, 1989). No hybridization between the Te^R determinants from the IncPα and IncHII plasmids could be detected, suggesting that the two determinants are not closely related.

3.4 Tn*1000* insertion mutagenesis of Te^R determinants.

The Te^R genes on pDT1558 and pDT1364 were localized by insertion mutagenesis with the transposon Tn*1000* (also called $\gamma\delta$). Mutants were selected by testing for loss of resistance to 50-64 µg/ml tellurite. The sites and orientations of the insertions were determined by comparing the double digest restriction endonuclease patterns obtained using the insertion mutants with the restriction maps of Tn*1000* (Figure

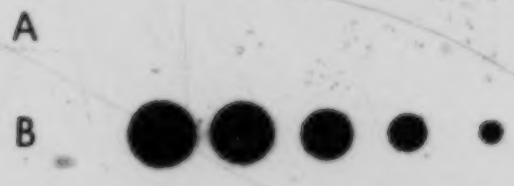


Figure 7. Lack of homology between IncPα and IncHII Te^R genes by dot blot hybridization.

Autoradiogram showing DNA-DNA hybridization between Te^R regions of RK2 (IncPα) and pHH1508a (IncHII). Fourfold dilutions of DNA fragments carrying the IncPα (A) or IncHII (B) Te^R region were applied to a nitrocellulose filter and hybridized with the ^{32}P-labelled Te^R region from pHH1508a.

8; Guyer, 1977) and either pDT1558 (Figure 5c) or pDT1364 (Figure 6a).

Out of a large number of Te^S Tn*1000* insertion mutants of pDT1558, 6 were isolated which had unique sites of insertion. The sites of insertion and orientations of Tn*1000* in these are shown in Figure 9 and listed in Table 4. The positions of insertion of the five mutants, pDT1558-1, 2, 3, 4, and 6 span a 1.75 kb region which is close to the 1.9 kb size for this Te^R determinant estimated by Taylor and Bradley (1987). In addition, a Tn*1000* insertion was obtained which mapped within the *kilA* region but also eliminated expression of tellurite resistance (pDT1558-5). The isolation of this mutant suggested that either *kilA* is required for expression of Te^R or the promoter for the Te^R gene(s) is located within the *kilA* gene.

Twenty random Te^S mutants of pDT1364 were obtained using Tn*1000* insertion mutagenesis. Restriction mapping of these mutants showed that insertions of Tn*1000* spanned a 1.25 kb region of pDT1364 from 3.3 to 4.55 kb on the restriction map (Figure 10; Walter and Taylor, 1989). Fifteen of these insertions were in the $\delta\gamma$ orientation and five were in the $\gamma\delta$ orientation (Table 5). This indicates that a minimum of approximately 1.25 kb of the 7 kb insert on this plasmid is involved in tellurite resistance.

Several Tn*1000* insertions into pDT1364 were found to have the same position and orientation (Table 5). These may have been different isolates of the same insertion mutant.

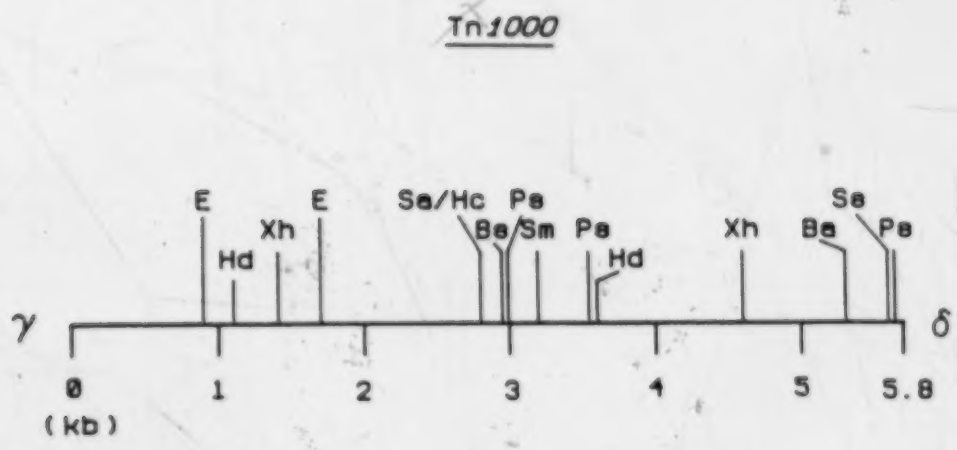


Figure 8. Restriction map of Tn*1000*.
A restriction map of the transposon Tn*1000* (also known as γδ) found on the F factor, is shown in the γδ orientation. Restriction sites which were used for mapping tellurite-sensitive insertion mutants are indicated (Guyer et al., 1978).

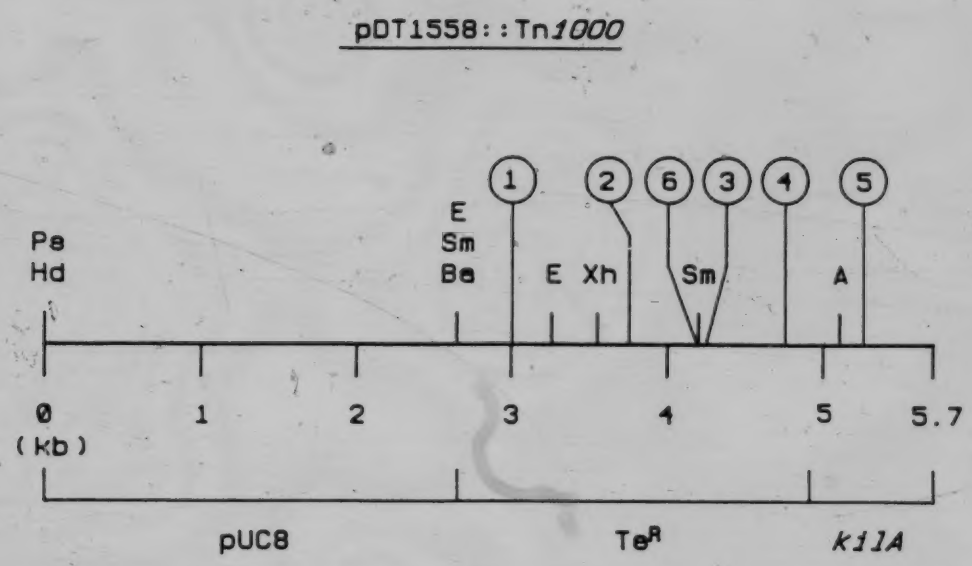


Figure 9. Tn*1000* insertions in the IncPα Te^R determinant. The arrows above pDT1558 indicate the positions of some Tn*1000* insertions which resulted in loss of tellurite resistance. Sites of insertion in plasmids pDT1558-1 to pDT1558-6 are indicated by numbers 1 to 6.

Table 4. Positions and orientations of Tn*1000* insertions into the IncPα Te^R determinant of pDT1558.

Tn*1000* insertion mutant (pDT #)	Position[1]	Orientation[2]
pDT1558-1 (1668)	3.0	γδ (—>)
pDT1558-2 (1671)	3.7	δγ (<—)
pDT1558-3 (1666)	4.2	γδ (—>)
pDT1558-6 (1670)	4.25	γδ (—>)
pDT1558-4 (1674)	4.75	δγ (<—)
pDT1558-5 (1665)	5.26	δγ (<—)

Notes:

[1]The positions on the restriction map of pDT1558 are shown in kilobases (Figure 5c).

[2]The orientation γδ indicates that Tn*1000* has inserted into pDT1558 in the orientation shown in Figure 7.

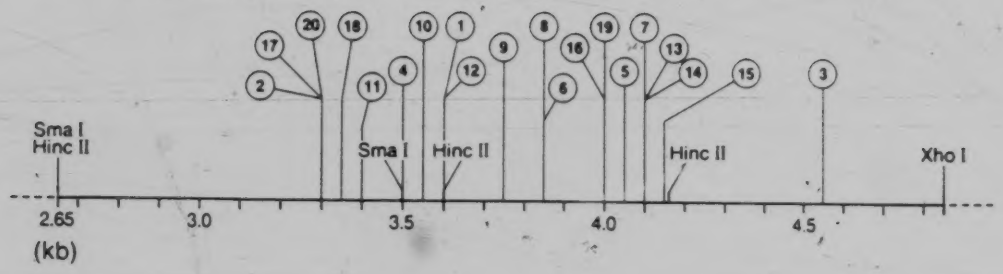


Figure 10. Tn*1000* insertions in the IncHII Te^R determinant. Restriction map of the Te^R region of pDT1364 showing the sites in which transposon Tn*1000* inserted to form Te^S mutants. Insertion mutants pDT1510 to pDT1529 are numbered 1 through 20 consecutively (pDT1364-1 to pDT1364-20).

Table 5. Positions and orientations of Tn*1000* insertions into the IncHII Te^R determinant of pDT1364.

Tn*1000* insertion mutant (pDT #)	Position[1]	Orientation[2]
pDT1364-2 (1511)	3.3	δγ (<—)
pDT1364-17 (1526)	3.3	δγ
pDT1364-20 (1529)	3.3	δγ
pDT1364-18 (1527)	3.35	δγ
pDT1364-11 (1520)	3.4	δγ
pDT1364-4 (1513)	3.5	γδ (—>)
pDT1364-10 (1519)	3.55	δγ
pDT1364-1 (1510)	3.6	γδ
pDT1364-12 (1521)	3.6	δγ
pDT1364-9 (1518)	3.75	γδ
pDT1364-8 (1517)	3.85	δγ
pDT1364-6 (1515)	3.85	δγ
pDT1364-16 (1525)	4.0	γδ
pDT1364-19 (1528)	4.0	γδ
pDT1364-5 (1514)	4.05	δγ
pDT1364-7 (1516)	4.1	δγ
pDT1364-13 (1522)	4.1	δγ
pDT1364-14 (1523)	4.1	δγ
pDT1364-15 (1524)	4.15	δγ
pDT1364-3 (1512)	4.55	δγ

[1]The positions are shown in kilobases on the restriction map of pDT1364 (Figure 6a).

[2]The orientation γδ indicates that the γ and δ ends of Tn*1000* (Figure 7) are located closer to the left and right ends, respectively, of the Te^R region of pDT1364 shown in Figure 6a.

Alternatively, these may indicate the presence of hot spots for insertion of Tn*1000*.

3.5 MICs of tellurite for Te^R and Te^S bacteria.

The minimum inhibitory concentrations (MICs) of potassium tellurite for *E. coli* strains carrying various Te^R and Te^S plasmids were determined using the agar dilution method. The MIC for *E. coli* (pHH1508a) was determined to be 512 µg/ml. The MIC for *E. coli* carrying the pUC8 clone pDT1364 was slightly lower at about 128 µg/ml. The MICs of the insertion mutants of pDT1364 were all between 0.25 and 1 µg/ml (Table 6) which is approximately the same as the MIC for the plasmid-free strains of *E. coli* (Taylor et al., 1988; Walter and Taylor, 1989).

The MIC for *E. coli* (RP4Te^R) was also 512 µg/ml. The MICs for *E. coli* carrying either of the pUC8 clones, pDT1366 or pDT1558, were slightly lower at 256 µg/ml. The MICs for most of the Tn*1000* insertion mutants of pDT1558 were 0.125 µg/ml (Table 6) which is slightly lower than that for the plasmid-free *E. coli*. An exception is the insertion mutant pDT1558-4 which is located near the right-most end of the region believed to be involved in tellurite resistance (Figure 9). This mutant had an MIC of 8 µg/ml which is intermediate between the MIC of *E. coli* (pDT1558) and that of the plasmid-free *E. coli* (Walter and Taylor, 1989).

Table 6. MICs of tellurite for Te^R and Te^S bacteria.

Plasmid[a]	MIC[b] (μg/ml K_2TeO_3)
pHH1508a	512
pDT1364	128
pDT1364-1 to pDT1364-20	0.25 to 1.0
RP4Te^R	512
pDT1366	256
pDT1558	256
pDT1558-1,2,3,5,6	0.125
pDT1558-4	8.0

[a]The MIC for plasmid-free strains of *E. coli* are 0.25 to 2 μg/ml.

[b]Minimum inhibitory concentration of potassium tellurite was determined by the agar dilution method.

Table 7. MICs under aerobic and anaerobic conditions.

E. coli strain	Aerobic MIC*	Anaerobic MIC*
J53-1	1-2	4
JM83	1	2-4
JM83 (pUC8)	2	4
JM83 (pDT1558)	~256	~256
JM83 (pDT1364)	~256	~256

*MICs were determined both by the agar dilution and broth dilution methods.

3.6 Effect of oxygen on the MIC for tellurite.

It was observed by Cooper and Few (1952) that aeration of cultures to remove reducing substances increased the rate of uptake of tellurite. Therefore, it was hypothesized that under anaerobic conditions there may be decreased uptake of K_2TeO_3 and thus an increased MIC. The MICs for tellurite of Te^R and Te^S bacterial strains were determined under aerobic and anaerobic conditions in broth and on agar plates to determine if oxygen had any effect on the sensitivity to tellurite (Table 7). Only a slight increase (about two-fold) in the level of resistance to tellurite of the sensitive bacterial strains *E. coli* JM83 and J53-1 was observed under anaerobic conditions. There was no difference in the MICs under aerobic and anaerobic conditions for strains carrying the Te^R plasmids pDT1558 or pDT1364.

3.7 Fertility inhibition properties of IncPα Te^R plasmids.

It was reported by Fong and Stanisich (1989) that RP1 (apparently identical to RP4 and RK2) decreases the mating frequency of the IncW plasmid R388 by a factor of approximately 10^5. One of the regions on this plasmid which appeared to be involved in fertility inhibition (*fiw*) was mapped to the same region as the Te^R determinant. Therefore, plasmids used in this study which carryied all or part of the Te^R plasmid RK2/RP4 were tested for their effect on the

Table 8. Fertility inhibition properties of Te^R plasmids.

Plasmid(s)[a]	Mating frequency of R388[b]
R388 alone	4.5×10^{-4}
R388 + RP4	$< 7.1 \times 10^{-7}$
R388 + pRK2102	3.6×10^{-5}
R388 + pDT1558	4.8×10^{-4}
R388 + pDT1558-1	5.3×10^{-4}
R388 + pDT1558-2	2.0×10^{-4}
R388 + pDT1558-3	3.9×10^{-3}
R388 + pDT1558-4	4.0×10^{-5}
R388 + pDT1558-5	6.9×10^{-4}

[a]R388 (Tp^R) and Te^R plasmids were maintained in donor strain (*E. coli* RG1900 [Rif^R]).

[b]The mating frequency of R388 from each strain to the recipient strain *E. coli* JE2571-1 (Nal^R) was determined by dividing the number of transconjugants (measured on NalTp plates) by the number of donors (measured on Rif plates).

mating frequency of R388 (Table 8). The presence of RP4 in the same cell as R388 decreased the mating frequency of R388 by more than 10^3 times. Cells carrying both R388 and pRK2102, which has the entire Te^R region of RK2/RP4, had only a ten-fold decrease in the mating frequency. The mating frequency was not decreased significantly by pDT1558 or most of its insertion mutants except pDT1558-4, which decreased the mating frequency by about 10-fold. This plasmid is only partially Te^R and has Tn*1000* inserted between *kilA* and the tellurite resistance genes (described below). Thus the association between the *fiw* and Te^R phenotypes could not be confirmed in this study.

3.8 Phage inhibition properties of IncHII Te^R plasmids.

The relationship between phage inhibition (*phi*) and tellurite resistance on the plasmid pHH1508a was examined by measuring the ability of the bacteriophage lambda to produce plaques on various bacterial strains with or without parts of the plasmid pHH1508a. The efficiency of plating with phage λ was determined in comparison with *E. coli* JM83. Both pHH1508a and its derivative pDT1178 reduced the plaquing efficiency of λ by more than 10^6 times (Table 9). In contrast, *E. coli* carrying pDT1364 had an efficiency of plating with phage λ which was equal to that of JM83 alone indicating that the *phi* phenotype was not expressed by this plasmid.

Table 9. Phage inhibition properties of IncHII Te^R plasmids.

E. coli (Plasmid)	Number of plaques[a] Dilutions undil	10^{-2}	10^{-4}	10^{-6}	10^{-8}	Efficiency of plating with phage λ[b]
JM83	+++[c]	++[d]	23	1	-	1
J53-1 (pHH1508a)	-	-	-	-	-	$<10^{-6}$
J53-1 (pDT1178)	-	-	-	-	-	$<10^{-6}$
JM83 (pDT1364)	+++	++	21	-	-	1
JM107 (pUC8)	+++	+++	+++	168	2	1.4×10^3

[a]Plaque counts are averages from two or three plates.

[b]Efficiency of plating with phage λ was determined by dividing the number of plaques obtained with the test strain by the number of plaques obtained with JM83.

[c]+++ complete destruction of cell layer

[d]++ too many plaques to count

E. coli JM107 carrying pUC8 alone had an increased plating efficiency with phage λ. The reason for this is not clear, however, it may be related to a different sensitivity of the different host strains of *E. coli* to phage λ.

3.9 Detection of plasmid-encoded proteins by *in vitro* transcription-translation.

The proteins encoded by the Te^R plasmids pDT1558 and pDT1364 were expressed using an *in vitro* transcription-translation system. Both pDT1558 (Figure 11; Walter and Taylor, 1989) and pDT1366 (not shown) were each found to encode a 40 kilodalton (kDa) polypeptide which was not expressed by pUC8. Coincidentally, the transposon Tn*1000* also produces a protein of about 40 kDa in size, as well as several other proteins (Taylor et al., 1987). Because of this transposon-encoded protein, it was not possible to determine if the insertion mutant pDT1558-4, which expressed an intermediate level of resistance to tellurite, still expressed the 40 kDa Te^R protein. No unique polypeptides were detected with either pDT1558-4 or pDT1558-1 in comparison to other insertion mutants (not shown). However, the Te^S Tn*1000* insertion mutants pDT1558-2 and pDT1558-3 produced proteins of 37 and 32 kDa, respectively (Figure 11). These polypeptides may be truncated mutants of the 40 kDa protein formed by insertion of a stop codon coded by Tn*1000* into the reading frame of the Te^R protein.

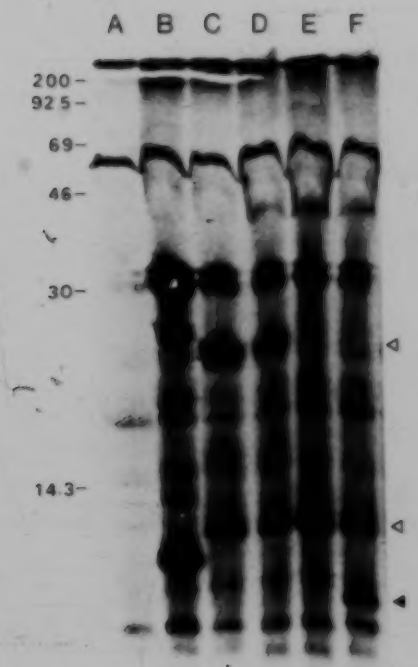
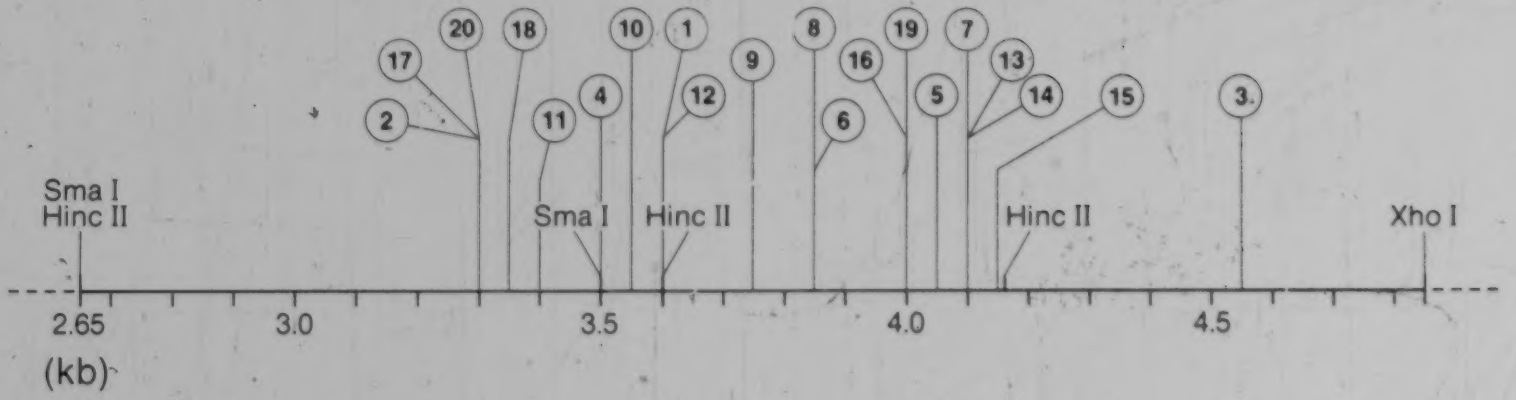


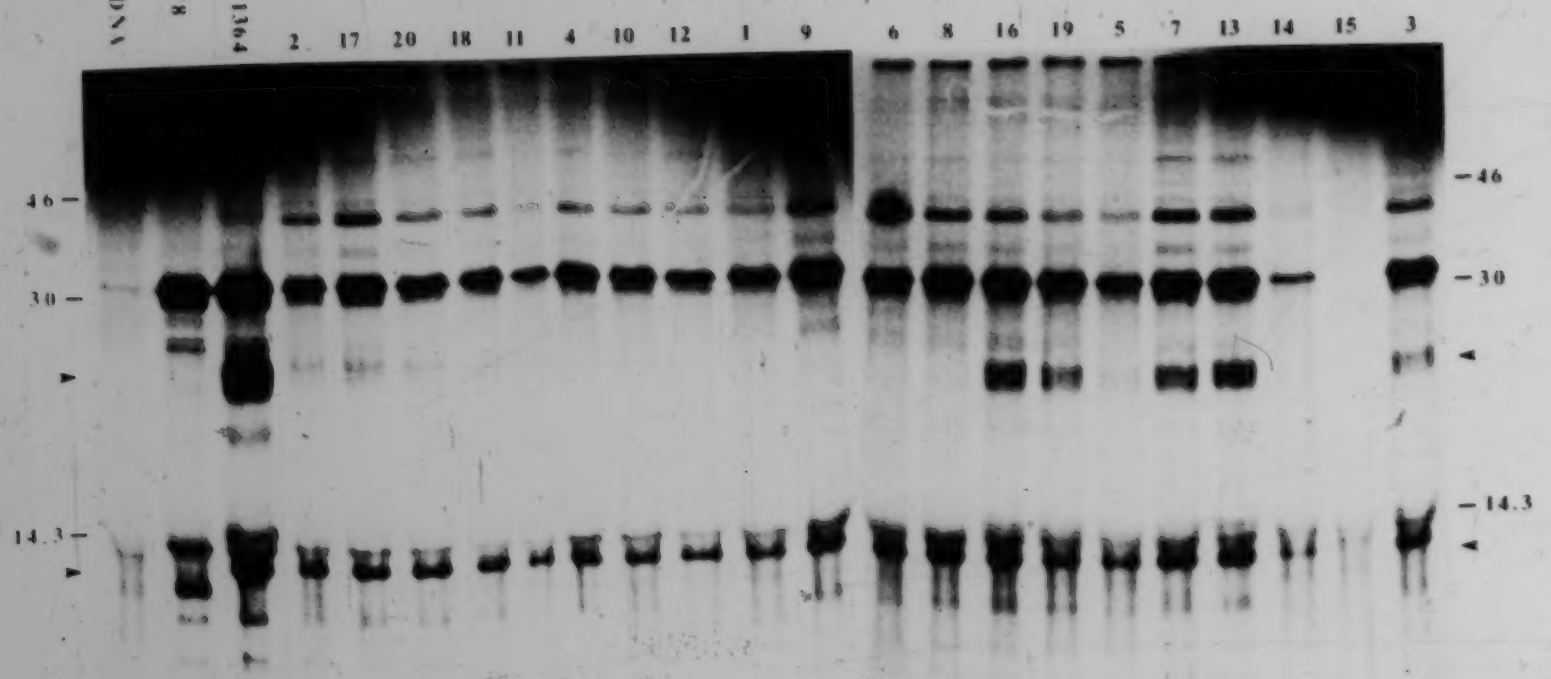
Figure 11. Expression of IncPα Te^R proteins in an *in vitro* transcription-translation system.

Fluorogram showing proteins expressed by IncPα Te^R clones and Te^S derivatives in an *in vitro* transcription-translation system. Lanes: (A) No DNA added; (B) pUC8; (C) pDT1558; (D) pDT1558-3; (E) pDT1558-2. The open triangle indicates the 40 kDa polypeptide expressed by pDT1558 but not by pUC8. The closed triangles indicate the positions of the mutant proteins expressed by two Tn*1000* insertion mutants. The molecular masses and positions of the standard proteins are indicated to the left in kilodaltons.

Figure 12. Proteins expressed by Tn*1000* insertion mutants of pDT1364.

Autoradiogram showing proteins expressed by the Tn*1000* insertion mutants of pDT1364 in an *in vitro* transcription-translation system. The first three lanes contain the no DNA control, pUC8, and pDT1364 as indicated. The next twenty lanes show proteins expressed by each of the insertion mutants pDT1364-1 through 20 (indicated by number above lane) loaded in the order of their positions of insertion in pDT1364 (Figure 10 and below). The 23 kDa and 12 kDa proteins expressed by pDT1364 are indicated by arrows. The sizes of molecular weight standards are shown on both sides in kilodaltons.




no DNA
pLC8
pDT1364
2
17
20
18
11
4
10
12
1
9
6
8
16
19
5
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30
14.3
46
30
14.3

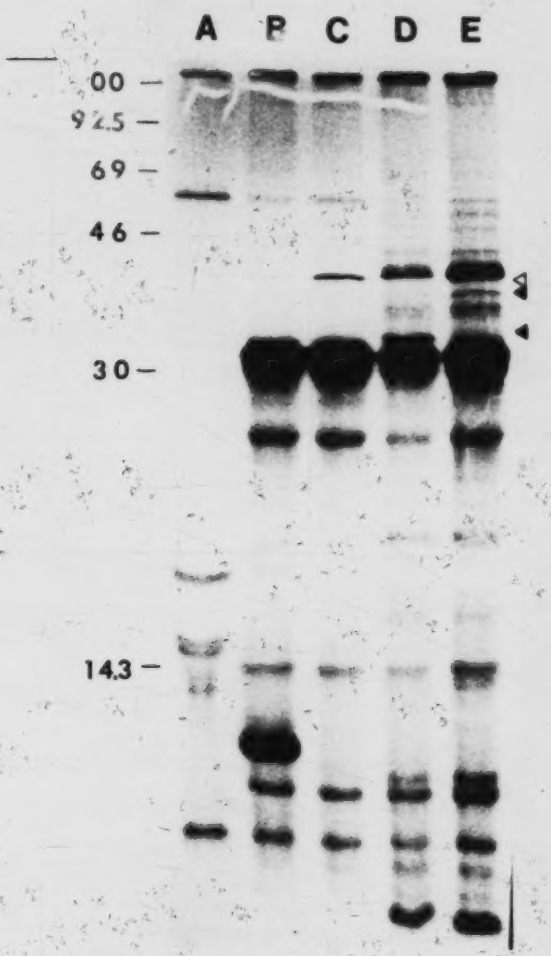

Figure 13. Unique proteins expressed by pDT1364 in an *in vitro* transcription-translation system.

Fluorogram showing proteins encoded by IncHII Te^R clones and Te^S derivatives in an *in vitro* transcription-translation system. Lanes: (A) no DNA added; (B) pUC8; (C) pDT1364; (D) pDT1364-17; (E) pDT1364-6; (F) pDT1364-1. Open triangles indicate the 23 and 12 kDa polypeptides expressed by pDT1364 but not by pUC8. The closed triangle indicates the mutant protein expressed by Tn*1000* insertion mutant pDT1364-1. The molecular masses and positions of the standard proteins are indicated to the left in kilodaltons.

Using the transcription-translation system, pDT1364 was found to encode two polypeptides of 23 kDa and 12 kDa which were not expressed by pUC8 (Figure 12, 13; Walter and Taylor, 1989). The Te^S Tn*1000* insertion mutants of pDT1364 were examined using this system and the proteins expressed by three of these are illustrated in Figure 14. Like pDT1364-6, many of the Tn*1000* insertion mutants did not express the 23 kDa polypeptide. One Tn*1000* insertion mutant, pDT1364-1, did not express the 23 kDa polypeptide but instead expressed a 9.5 kDa polypeptide. This unique polypeptide appears to be a truncated mutant of the 23 kDa protein. No unique polypeptides could be detected in the other insertion mutants (Figure 12). The 12 kDa polypeptide appeared to be expressed in all of the Te^S Tn*1000* insertion mutants, suggesting that this protein may not be involved in tellurite resistance.

The sizes of proteins produced by the three Tn*1000* insertion mutants, pDT1364-6, -1, and -17 suggest that the Te^R gene coding for the 23 kDa protein is transcribed from right to left on the map of pDT1364 (Figure 14). The size of DNA required to code for a 23 kDa protein (approx. 0.6 kb) is much smaller than the size of the region determined to be involved in tellurite resistance by Tn*1000* insertion mutagenesis. This suggests that there may be other encoded proteins which have not been detected using this system.

Proteins produced by Tn*1000* Insertion Mutants

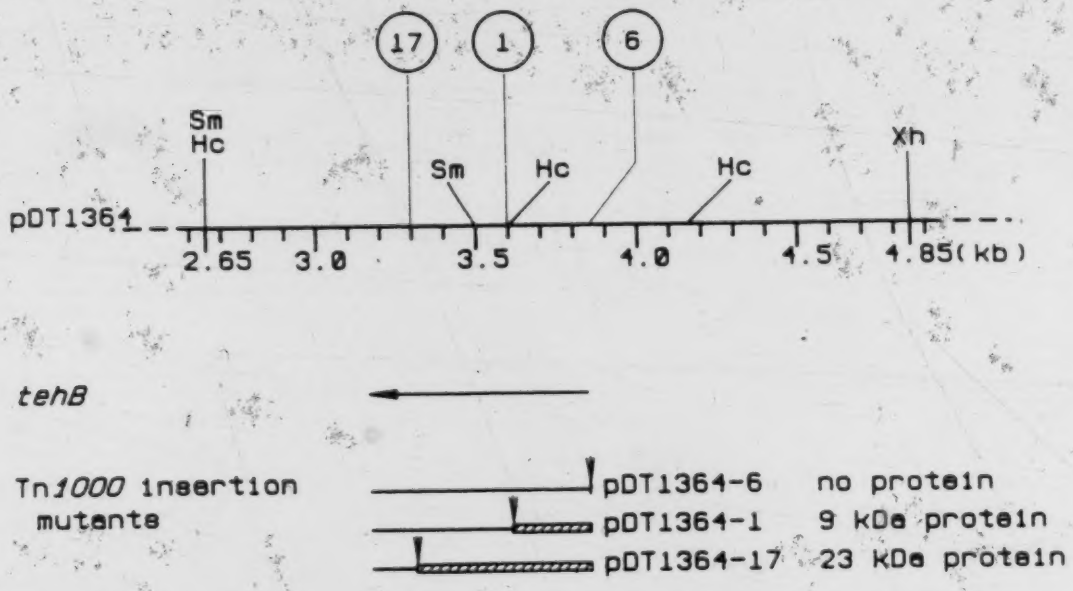

Figure 14. Truncated proteins produced by pDT1364::Tn*1000*. The points of insertion of Tn*1000* in pDT1364-1, pDT1364-6, and pDT1364-17 are shown. The sites of insertion in pDT1364-1 and pDT1364-6 were determined by DNA sequencing (see Figure 39). The relative sizes of mutant TehB proteins expressed by these three plasmids in an *in vitro* transcription-translation system are indicated by cross-hatched rectangles.

3.10 Preparation of β-galactosidase fusions.

The mini-Mu transpositional phages, mini-MudI and mini-MudII, carry most of the gene for β-galactosidase, a 116 kDa cytoplasmic protein, as well as resistance to kanamycin (Figure 15). Transposition of the phages mini-MudI and mini-MudII into a gene can result in transcriptional or translational *lac* fusions, respectively, if the insertion is in the correct orientation, and, in the case of mini-MudII, if it inserts in the correct reading frame (Castilho et al., 1984).

Thirteen Te^S mutants of pDT1555 were isolated by insertion of the transcriptional fusion phage mini-MudI. These were mapped to 10 different positions within the 3 kb insert of pDT1555 (Figure 16). The orientation of insertion of mini-MudI in each mutant indicated that transcription was occurring from right to left on the map of pDT1555. All transcriptional fusions resulted in the inactivation of the Te^R determinant (MIC $\leq$ 1 µg/ml potassium tellurite, whereas *E. coli* (pDT1555) had an MIC of 256 µg/ml). A total of seven mutations mapped within the *kilA* gene.

Similar results were obtained with the translational fusions using mini-MudII (Figure 15), in that of 18 mutants isolated, 11 mutations were mapped in the *kilA* gene (Figure 16). Again, the direction of insertion indicated translation in only one direction. However, not all of the mini-MudII inserts showed a complete loss of Te^R. Three mutants, in

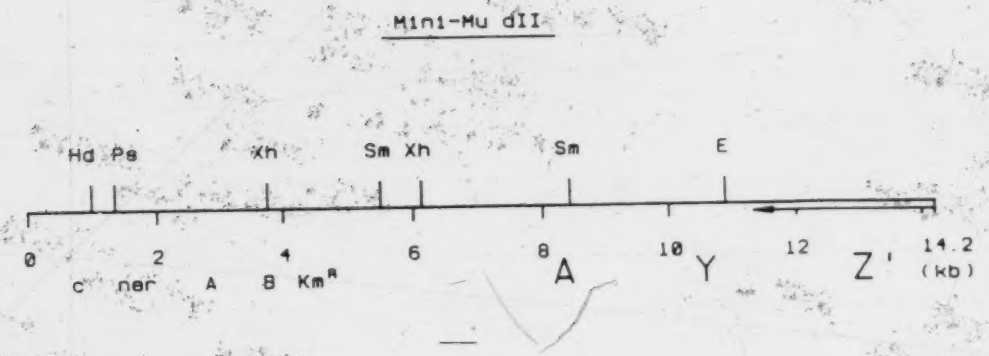

Figure 15. Restriction map of mini-MudII.
Restriction endonuclease sites and relevant genes on the *lac* fusion phage mini-MudII are indicated (Castilho et al., 1984; D. E. Taylor, unpublished data).

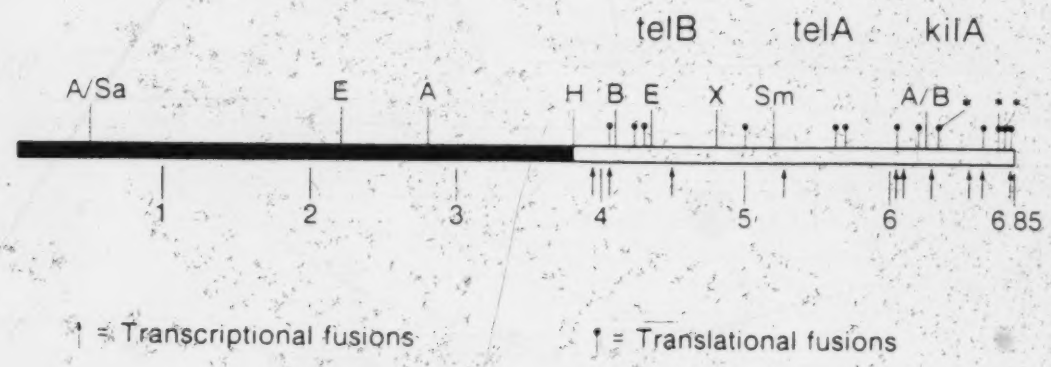

Figure 16. Fusions of *lacZ* to the IncPα Te^R region.
Mini-Mu insertions into pDT1555 which eliminate or reduce resistance to potassium tellurite (Te^R). Plasmid pDT1555 contains the tellurite resistance region of RK2 inserted into pACYC184 (solid line). Lower symbols (↑) denote transcriptional fusions in which a mini-MudIlac phage has inserted to inactivate Te^R. Upper symbols (↑) denote translational fusions in which a mini-MudIIlac phage inserted either to inactivate Te^R (MIC < 1 µg/ml) or to reduce the level of resistance (*) to an MIC of Te of 16 µg/ml.

which the inserts were at or close to the beginning of the *kilA* gene, specified low level resistance to Te (MIC = 16 µg/ml). Since insertion of mini-Mu into *kilA* eliminated or reduced the level of tellurite resistance, it appears that the promoter for the Te^R genes may be close to or identical to the *kilA* promoter. The isolation of active β-galactosidase transcriptional and translational fusions to *kilA* indicated that transcription and translation of the *kilA* gene is occurring.

Four unique lac^+Te^S insertions of mini-MudII into pDT1364 were obtained (Figure 17). The direction of insertion indicated that transcription and translation is occurring from right to left as shown on the map of this plasmid. These insertions were found in several different restriction fragments suggesting that they are located in several different positions within the Te^R gene(s).

3.11 Preparation of alkaline phosphatase fusions.

The transposon Tn*phoA* carries the gene for alkaline phosphatase, a 94 kDa periplasmic protein, minus its signal sequence, in the left end of Tn*5* (Figure 18; Manoil and Beckwith, 1985; Boquet et al., 1987). Insertions of Tn*phoA* were constructed in order to determine if any of the proteins encoded by this region were membrane-associated (Manoil and Beckwith, 1985).

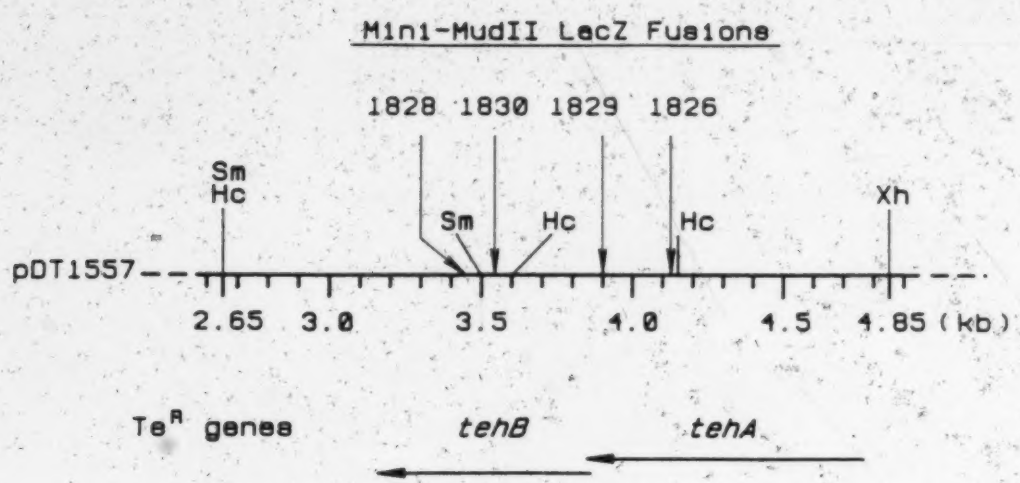

Figure 17. Fusions of *lacZ* to the IncHII Te^R region.
The positions of the mini-MudII *lac* fusion phage in four insertions into pDT1557 (IncHII Te^R) which resulted in expression of β-galactosidase and loss of tellurite resistance are shown.

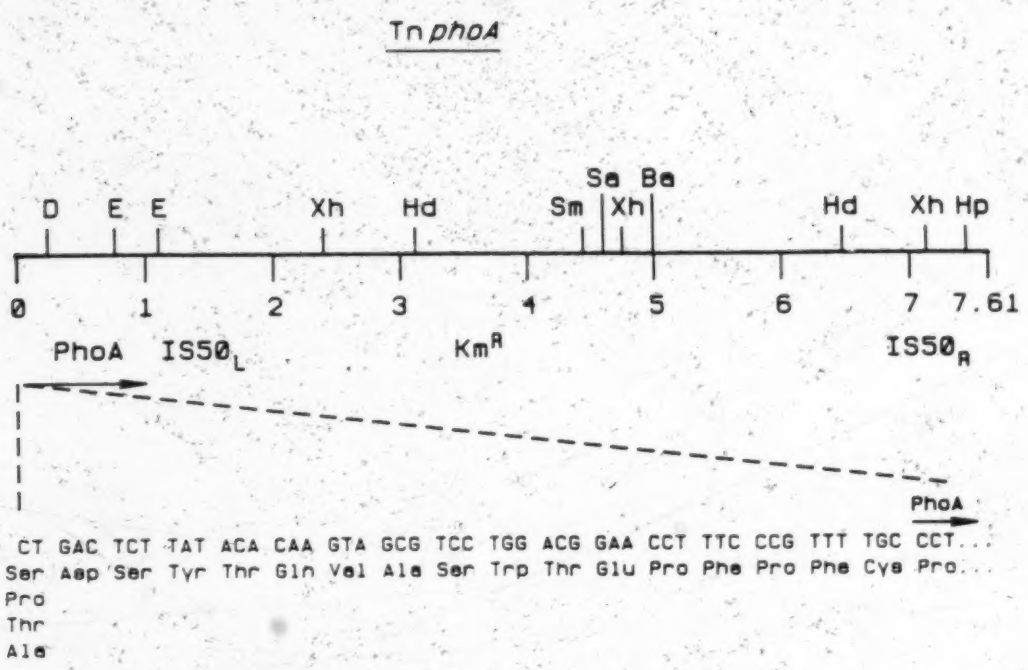
Tn*phoA*
D E E Xh Hd Sm Se Xh Ba Hd Xh Hp
0 1 2 3 4 5 6 7 7.61
PhoA
IS50L
KmR
IS50R
PhoA
CT GAC TCT TAT ACA CAA GTA GCG TCC TGG ACG GAA CCT TTC CCG TTT TGC CCT...
Ser Asp Ser Tyr Thr Gln Val Ala Ser Trp Thr Glu Pro Phe Pro Phe Cys Pro...
Pro
Thr
Ala

Figure 18. Restriction map of Tn*phoA*.

Tn*phoA* contains the coding sequence for alkaline phosphatase (minus its signal sequence) inserted into the left end of Tn*5*. The positions of restriction sites within Tn*phoA* which were used in mapping insertion mutants are shown (Boquet et al., 1987). The nucleotide sequence and predicted amino acid sequence of the left end of Tn*phoA* is shown as well as four possible amino acids that could be encoded in a fusion protein (Manoil and Beckwith, 1985).

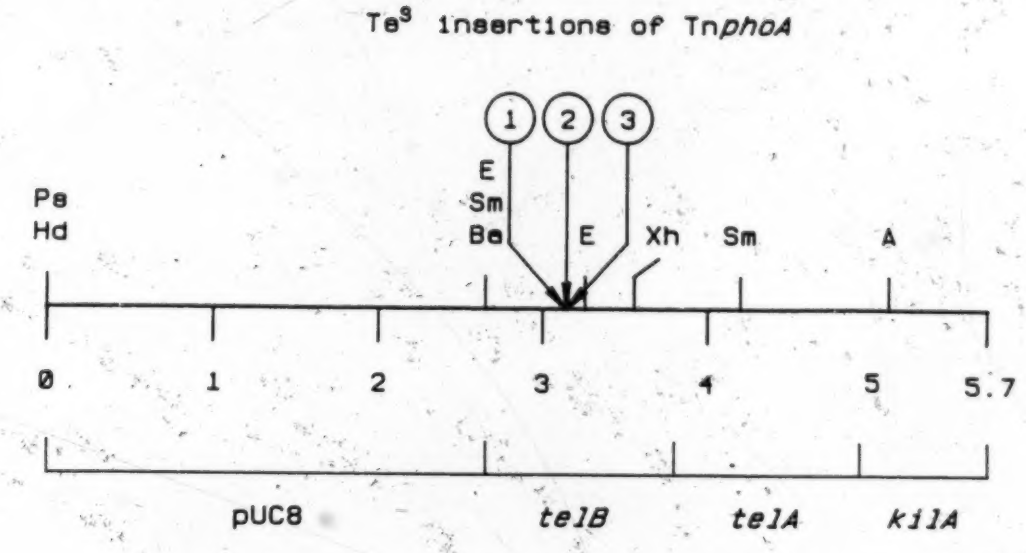

Figure 19. TnphoA insertions into pDT1558.
The site of insertion of Tn*phoA* into pDT1558 in three independent experiments is shown. Each insertion resulted in the expression of alkaline phosphatase and the elimination of tellurite resistance.

Three independently isolated insertions into the IncPα Te^R region on pDT1558 were obtained which eliminated the expression of tellurite resistance and allowed the expression of alkaline phosphatase as detected by the formation of blue colonies on media containing XP. Each of these Tn*phoA* insertions was mapped to the same site within the *telB* gene (Figure 19). The junction point of one of these insertions was sequenced and found to be in the correct reading frame for fusion with the TelB protein (Figure 18; see section 3.11). This result suggests that the Te^R determinant from RK2 encodes a membrane or periplasmic protein.

Six insertions of Tn*phoA* into pDT1364 were obtained which expressed alkaline phosphatase as detected by the formation of blue colonies on media containing XP (Figure 20). Three insertions did not affect expression of Te^R. These were mapped to positions far removed from the Te^R region as identified by Tn*1000* insertion mutagenesis. The identity of the genes in this region is unknown. Three other insertions of Tn*phoA* in pDT1364 did eliminate resistance to tellurite. These were all mapped to a region close to the *Xho*I site on this plasmid (Figure 20). These Tn*phoA* insertions were obtained in three separate experiments, suggesting that there may be a hot spot for insertion of Tn*phoA* in this region. The site of insertion in one of these mutants was determined by DNA sequencing and determined to be in the correct reading frame for fusion to one of the Te^R proteins (Figure 18;

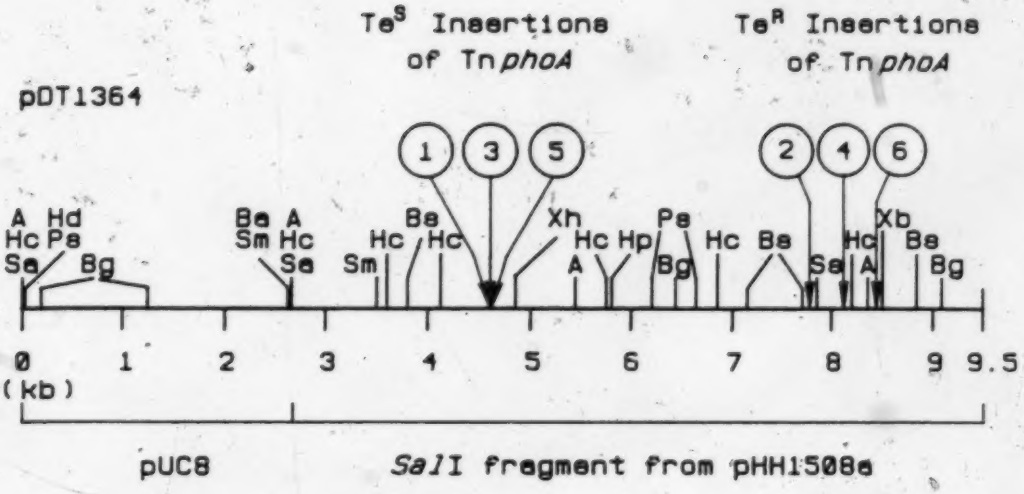

Figure 20. TnphoA insertions into pDT1364.
The sites of insertion of Tn*phoA* into pDT1364 in six independent experiments are shown. Each insertion resulted in the expression of alkaline phosphatase. Insertions 1, 3, and 5 resulted in loss of tellurite resistance whereas insertions 2, 4, and 6 had no effect on expression of tellurite resistance.

Section 3.17). This result suggests that the Te^R determinant from pHH1508a also encodes a membrane or periplasmic protein.

3.12 DNA sequencing analysis of the IncPα Te^R region.

The nucleotide sequence of the Te^R region of RK2Te^R was determined by subcloning fragments of DNA from pDT1558 or its Tn*1000* insertion mutants into the bacteriophages M13mp18 or M13mp19 (Figure 21). Portions of the sequence were determined by double-stranded sequencing of pDT1558 or its insertion mutants using specially designed oligonucleotide primers.

Potential open reading frames (ORFs) were identified by localization of probable initiation codons (ATG) and termination codons (TAA, TAG, TGA) (Figure 22). Three large ORFs were thus identified (Figure 23). The first ORF, extending from nucleotide (nt) coordinates 75 to 845 (Figure 22), can encode a protein of 257 amino acids (aa) in length with a predicted molecular mass of approximately 28,391 daltons. This ORF corresponds to the *kilA* gene which has been localized to this region (Figurski et al., 1982).

The promoter and operator sequences for *kilA* have already been identified (Young et al., 1985). The hexanucleotide TTGACG is found at nt residues 2 to 7 in the sequence (Figure 22). This matches the *E. coli* -35 consensus sequence (TTGACA; Rosenberg and Court, 1979) in 5 out of 6 positions. An AT-rich hexamer (TAAACT) corresponding to the -10 portion

DNA Sequencing Strategy for IncPα Tellurite Resistance Genes

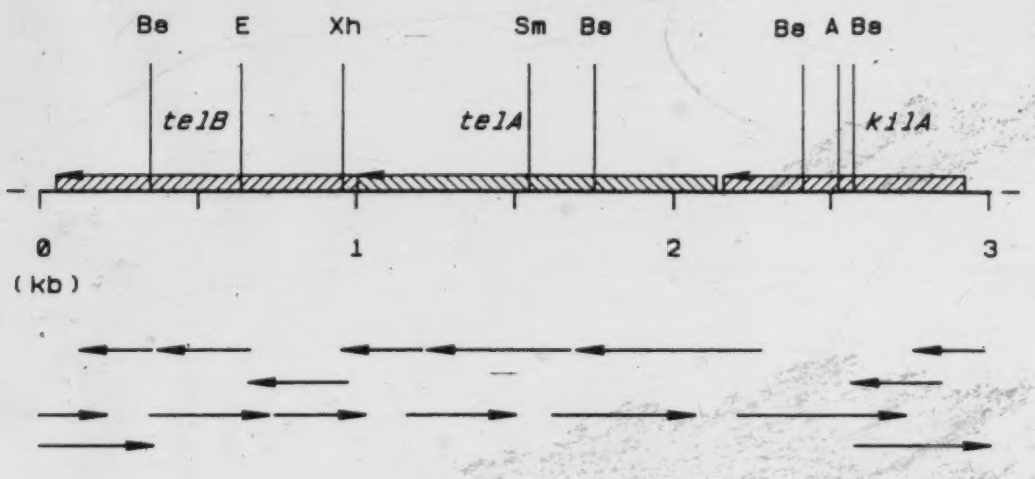

Figure 21. Sequencing strategy for the IncPα Te^R genes. The restriction endonuclease map of the Te^R region of RK2Te^R contained in pDT1558 is shown. Arrows underneath indicate the direction and length of individual regions sequenced. Open reading frames for the genes *kilA*, *telA*, and *telB* are indicated in cross-hatching.

of the promoter (TATAAT) is located downstream at nt positions 26 to 31. There are 18 nt separating these two regions which is close to the optimal spacing (Rosenberg and Court, 1979). The sequence CAT, commonly found at the transcription initiation site, is found 6 nt downstream of the end of the proposed -10 region. The sequence GAGG found within the *E. coli* ribosome-binding sequence (AAGGAGGT) identified by Shine and Dalgarno (1974) is located 9 nt upstream of the proposed translational initiation codon for *kilA* (starting at nt 75 in Figure 22).

A second open reading frame extending from nt coordinates 866 to 1999 can encode a protein of 378 aa with a predicted molecular mass of 42,130 daltons. This gene has been designated *telA* (tellurite-resistance gene A) since insertions of Tn*1000* or mini-Mu into this region eliminate Te^R. This ORF follows only 20 nt after the end of *kilA*. The sequence GGAG found within the Shine-Dalgarno ribosome-binding sequence is located upstream of the proposed start codon, with a separation of 6 nt. The site of insertion of Tn*1000* in the plasmid pDT1558-4 was located to a site 20 nt before the proposed start codon for *telA* by DNA sequencing. This plasmid confers an intermediate level of tellurite resistance upon its host probably due to expression of the *telA* and *telB* genes from a promoter within Tn*1000*.

A third ORF extending from nt coordinates 1999 to 2949 was identified and named *telB* since transposon insertions in this region also result in sensitivity to tellurite (Figure

Figure 22. Nucleotide and predicted amino acid sequence of the Te^R region of RK2.

The nucleotide sequence of the coding strand of the *kilA*-Te^R region of RK2Te^R is shown. The deduced amino acid sequences of the three largest open reading frames are shown underneath. The ORFs have been named *kilA*, *telA*, and *telB*. Hexanucleotides within the probable promoter are indicated by "-35" and "-10". Possible ribosome binding (Shine-Dalgarno) sequences are marked "SD". The sites complementary to the oligonucleotides made for RNA primer extension are marked "Primer 1" and "Primer 2". mRNA start sites determined by primer extension from primers 1 and 2 are marked "mRNA-1" and "mRNA-2", respectively. Points of insertion of Tn*1000* and Tn*phoA* in various insertion mutants are also indicated with an arrow. Differences between sequences from RK2Te^R and RK2Te^S are indicated as follows: lower-case letters above the sequence indicate missense mutations (nt 2371); underlined bases in the sequence at nt 1282, 2522, and 2966 are not found in the Te^S sequence (ie. frame-shift mutations).

-35
-10
mRNA-2
SD
MetGluGluGlnSerV alAsnMetAlaArgLeuLysGlyGluValL (klIA)
euProAlaLeuPheAlaSerProAlaThrI leGlyGluTyrGlyAlaGlyIleAspGlyA
Tn1000 (1558-5)
laAspSerLeuAsnGluLeuSerAsnLeuM etGluHisGlyAlaValAlaAlaLeuAlaA
spLysIleSerGlnIleValAlaLysLeuA laAspAlaAspProArgLysIleAlaGluL
mRNA-1
ysProThrTrpPheGluLysMetLeuGlyA rgGluValGluArgGlnValArgTyrGlnV
alAlaArgLysThrLeuAspGlnLeuLeuA spGluAlaGluGlyValAlaGlnArgValA
Bs
Primer 2
A
rgAspThrLeuArgAlaLeuAspAspMetL euAsnThrHisGluAlaGluValAspArgL
euArgAlaTyrIleGlnAlaGlyArgGluP heLeuAspGluAsnProGluAlaGlyAlaA
Bs
laLysAlaGlyValIleGluPheAspLysP roArgGluArgPheAlaArgLysLeuAlaA
snLeuAlaThrLeuMetAlaSerHisGluM etSerValThrGlnMetLysLeuThrArgA
laGlnAlaValAspMetLeuAspArgPheS erGluThrAlaSerValLeuValProValT
rpArgGlnHisThrLeuAlaLeuIleThrT hrLysAsnMetAsnProAlaMetValAlaG

```
      790         800         810         820         830         840
AGGCGGCCAAAGCTCACCAGGCGCTCATGC GGAGCCTTTCGCAGAGCCTGGAAGGCATCA
luAlaAlaLysAlaHisGlnAlaLeuMetA rgSerLeuSerGlnSerLeuGluGlyIleA
Tn1000(pDT1558-4)
                    SD
      850         860         870         880         890         900
ACCAATAACACGGCGGGAGAACCCTATGAA CGCACTGAAAACGACGCACGACGCCAAGGC
snGln                       MetAs nAlaLeuLysThrThrHisAspAlaLysAl (telA)
      Primer 1
<     910         920         930         940         950         960
CCCTATCGTCGCCTTCGACATGACCCCGGC AACCCTGCGCGAGCTGGGCTTGCAGGAAAG
aProIleValAlaPheAspMetThrProAl aThrLeuArgGluLeuGlyLeuGlnGluSe

      970         980         990        1000        1010        1020
CGACGTGCCGGAAGTCCATGCGGTCGCGCA GCGGATCGAGGTCGGCAGTCCGCAGACCGT
rAspValProGluValHisAlaValAlaGl nArgIleGluValGlySerProGlnThrVa

     1030        1040        1050        1060        1070        1080
TGCCGAGTTCGGCCGCGACGTGGCCGAGCA CACGTCCCGCTACGCCGATAGCCTGCTGGA
lAlaGluPheGlyArgAspValAlaGluHi sThrSerArgTyrAlaAspSerLeuLeuAs

     1090        1100        1110        1120        1130        1140
CCAGGTGCGCAACAGCGACCTGGACGAAGC AGGCGAGAAACTGACCCAGGTTGTCGCCAA
pGlnValArgAsnSerAspLeuAspGluAl aGlyGluLysLeuThrGlnValValAlaLy

     1150        1160        1170        1180        1190        1200
GGCCCGTTCCCTGAACGTCGGCCCTTTGTC CGACAACCGTTCCCGCCTGCCCCTGATTGG
sAlaArgSerLeuAsnValGlyProLeuSe rAspAsnArgSerArgLeuProLeuIleGl
                                                  Bs
     1210        1220        1230        1240        1250        1260
CCCGCTGATCGACCGCTTCCGCGTCCGTTC GACGGGCTTCATGGCGCGCTTCGACACGAC
yProLeuIleAspArgPheArgValArgSe rThrGlyPheMetAlaArgPheAspThrTh

     1270        1280        1290        1300        1310        1320
CCGCGAGCAGATCGAACACCTGGTCAGCGA AGTGCAGACCACCCAGCAAGGCATCGCGCA
rArgGluGlnIleGluHisLeuValSerGl uValGlnThrThrGlnGlnGlyIleAlaGl

     1330        1340        1350        1360        1370        1380
GCGCAATGCCTCGCTCGACGAAATGTTCGC AGCCGTGCGCGAGGAACACCGCCTTCTTGG
nArgAsnAlaSerLeuAspGluMetPheAl aAlaValArgGluGluHisArgLeuLeuGl

     1390        1400        1410        1420        1430        1440
CGTCCACATCGCGGCCGGCAAGGTCCGCCT TGCCGAGCTGCGCGAGCAGGCCGAGGGTCT
yValHisIleAlaAlaGlyLysValArgLe uAlaGluLeuArgGluGlnAlaGluGlyLe
                          Sm
     1450        1460        1470        1480        1490        1500
GCGCGGCAATGTCGGGAACGACCCGGGCCG CGTGCAGGAGCTGGCCGACCTCGATGCGAT
uArgGlyAsnValGlyAsnAspProGlyAr gValGlnGluLeuAlaAspLeuAspAlaMe

     1510        1520        1530        1540        1550        1560
GGTTGCCAACCTGGACAAGCGCATCGGCGA CCTGATCGCCTTGCAACATTCGGCCATGCA
tValAlaAsnLeuAspLysArgIleGlyAs pLeuIleAlaLeuGlnHisSerAlaMetGl
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      1570       1580       1590       1600       1610       1620
GAGCCTGCCGACCATCCGCATGATCCAGGC CAACAACCAGATGCTGGTCGATAAATTCCA
nSerLeuProThrIleArgMetIleGlnAl aAsnAsnGlnMetLeuValAspLysPheHi

      1630       1640       1650       1660       1670       1680
CACCATCCGCGAAATCACCGTGCCGGCGTG GAAGCGGCAATTCATGCTGGCCTTGAGCCT
sThrIleArgGluIleThrValProAlaTr pLysArgGlnPheMetLeuAlaLeuSerLe

      1690       1700       1710       1720       1730       1740
CAACGAGCAGAAGAACGCCGTCGAACTGGC CACGGCCATCGACGACACCACCAACGACCT
uAsnGluGlnLysAsnAlaValGluLeuAl aThrAlaIleAspAspThrThrAsnAspLe

      1750       1760       1770       1780       1790       1800
GATGAAGCGCAATGCGGCCCTGCTGCATCG CACGTCCGTCGAGACGGCGAAGGAGAACCA
uMetLysArgAsnAlaAlaLeuLeuHisAr gThrSerValGluThrAlaLysGluAsnGl

      1810       1820       1830       1840       1850       1860
ACGCCTGGTGATCGACGTGGACACGCTCAA GCAGGTTCAGACGACGCTCATCAAGACCGT
nArgLeuValIleAspValAspThrLeuLy sGlnValGlnThrThrLeuIleLysThrVa
Tn1000 (pDT1558-2)
      1870       1880       1890       1900       1910       1920
CGAGGACGTTATTCGCATCCAGCAGGAAGG CGTGCAGAAGCGCAAGGATGCCGAGAAGCA
lGluAspValIleArgIleGlnGlnGluGl yValGlnLysArgLysAspAlaGluLysGl

      1930       1940       1950       1960       1970       1980
GATCGCCGCAATGCGTGGCGATCTTCAAGC CAAGCTGACCCGCCAGCCCGTGCGCGAGCT
nIleAlaAlaMetArgGlyAspLeuGlnAl aLysLeuThrArgGlnProValArgGluLe
          SD
      1990       2000       2010       2020       2030       2040
GGCCCAACAGGAGTCCGTATGAATGCCACA AACACCGATGTTTTCGCCCAGGTAGGCGGC
uAlaGlnGlnGluSerVal
                  MetAsnAlaThr AsnThrAspValPheAlaGlnValGlyGly (tolB)
   Xh
      2050       2060       2070       2080       2090       2100
CTCGAGGCCCGAGGCGCGAAGATGAAGAAG CGGGGCACCCGCTTCCTCATCGCGGCGCTG
LeuGluAlaArgGlyAlaLysMetLysLys ArgGlyThrArgPheLeuIleAlaAlaLeu

      2110       2120       2130       2140       2150       2160
GCAGTCCTTGCCATTGCCGGGATCGGGGCA GTAACGGGATGGGCGATCAGCCCGAGCGCG
AlaValLeuAlaIleAlaGlyIleGlyAla ValThrGlyTrpAlaIleSerProSerAla

      2170       2180       2190       2200       2210       2220
ACGCCCGGAAGCATTGACGTGCCGCAGGTG CTGGCATCGACATTCAGCGACCAGGTGCCG
ThrProGlySerIleAspValProGlnVal LeuAlaSerThrPheSerAspGlnValPro

      2230       2240       2250       2260       2270       2280
GGCAGTGAGGGCGGCGGCCTGGGTGGCGGC CTGCCCTTCACTTCGGCCGTCGGGGCATTC
GlySerGluGlyGlyGlyLeuGlyGlyGly LeuProPheThrSerAlaValGlyAlaPhe

      2290       2300       2310       2320       2330       2340
ACGGACTTCATGGCGGGGCCGGCAATTTTT ACCTTGGGCATTCTTGGCATAGTGGTCGCG
ThrAspPheMetAlaGlyProAlaIlePhe ThrLeuGlyIleLeuGlyIleValValAla
```

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                                   E
      2350       2360       2370  a      2380       2390       2400
GGTGCCGTGCTCGTGTTCGGGGGTGAATTC TGCGGGTTCGTGCGATCCGTCTGCATGATG
GlyAlaValLeuValPheGlyGlyGluPhe CysGlyPheValArgSerValCysMetMet

      2410       2420       2430       2440       2450       2460
GTGATAGCCGTCAGCATGATTTTCGTGTCG TCGAACTTGGTGAAGGGCATTCTCGGCGGC
ValIleAlaValSerMetIlePheValSer SerAsnLeuValLysGlyIleLeuGlyGly

      2470       2480       2490       2500       2510       2520
GATCACGACGCCGGCCCTGCGGAGCCTTCG CCGCGTGCGCGATTCATGGCGGCCGTGGAG
AspHisAspAlaGlyProAlaGluProSer ProArgAlaArgPheMetAlaAlaValGlu

      2530       2540       2550       2560       2570       2580
GCCAAGGATTTCGCGCGAGTGCAAGAGCTG ATCGAGGCGCGTGGAGCCAAGTCGGCGGCT
AlaLysAspPheAlaArgValGlnGluLeu IleGluAlaArgGlyAlaLysSerAlaAla
                                                          Bs
      2590       2600       2610       2620       2630       2640
GATTATGTCCTTGCGCAGCTCGCCGTGGCC GAAGGTCTGGACCGCAAGCCTGGTGCGCGC
AspTyrValLeuAlaGlnLeuAlaValAla GluGlyLeuAspArgLysProGlyAlaArg
                                  TnphoA
      2650       2660       2670       2680       2690       2700
GTCGTGGTCGGGAAAGCGGCGGGCAGCATG GCAATGCCGCCTGCGGCGCTGGGTTTTACG
ValValValGlyLysAlaAlaGlySerMet AlaMetProProAlaAlaLeuGlyPheThr
          Tn1000 (pDT1558-1)
      2710       2720       2730       2740       2750       2760
CCAAGGGGAGAAGCGGCATACGCCATCGAG CGGTCAGCCTATGGTGAGCCGAGGTCCAGC
ProArgGlyGluAlaAlaTyrAlaIleGlu ArgSerAlaTyrGlyGluProArgSerSer

      2770       2780       2790       2800       2810       2820
ATTGCGAAGCAGTACCAGCAGGAATGGAAC CGGAAGGCGGCGACCTGGTGGGCGATGGCC
IleAlaLysGlnTyrGlnGlnGluTrpAsn ArgLysAlaAlaThrTrpTrpAlaMetAla

      2830       2840       2850       2860       2870       2880
GGTGTGGCCGGCATCATCGGCGCGATCCTG GCGGCGGCGGCAACCGGCTTTGTTGGGCTG
GlyValAlaGlyIleIleGlyAlaIleLeu AlaAlaAlaAlaThrGlyPheValGlyLeu

      2890       2900       2910       2920       2930       2940
GCAGTGTCGATCCGCAACCGAGTGAAGCGC GTGCGCGACCTGTTGGTGATGGAGCCGGGT
AlaValSerIleArgAsnArgValLysArg ValArgAspLeuLeuValMetGluProGly

      2950       2960       2970       2980       2990       3000
GCAGAGCCATAAGCGGCAAGAGACGAAAGC CCGGTTTCCGGGCTTTTGTTTTGTTACGCC
AlaGluPro

      3010       3020
AAGGACGAGTTTTAGCGGCTAAAGGTG
```

22). This reading frame can code for a polypeptide of 316 aa in length with a molecular mass of 32,375 daltons.

The site of insertion of Tn*phoA* into the *telB* gene was determined by sequencing of the fusion junction and found to be immediately after nt residue 2680 within amino acid residue 228. Insertion was found to be in the correct reading frame for the production of a TelB-PhoA fusion protein (see Figure 18). The DNA sequence upstream of this site has some similarity to some of the hot-spot sites of insertion of Tn*phoA* identified by Gött and Boos (1988).

Because of the absence of sequences resembling a promoter before *telB*, it appears that *telA* and *telB* are transcribed as a single unit or operon. One nucleotide of the proposed start codon for *telB* overlaps with the last codon of *telA*, therefore it is predicted that this ORF would be translated immediately after translation of *telA* is completed, without dissociation of the ribosome. However, there is a potential ribosome-binding sequence, GGAG, six nt upstream of the proposed start codon for *telB*.

Another possible start codon for *telB* is located at nt residue 2062, shortly downstream of the start codon proposed in Figure 22. Upstream of this Met codon (separated by 7 nt) is the sequence GAGG which could act as a ribosome binding site. This potential start site is interesting because it is immediately followed by a 6 aa sequence containing four basic residues (LysLysArg.,Arg) then a long uncharged region. This

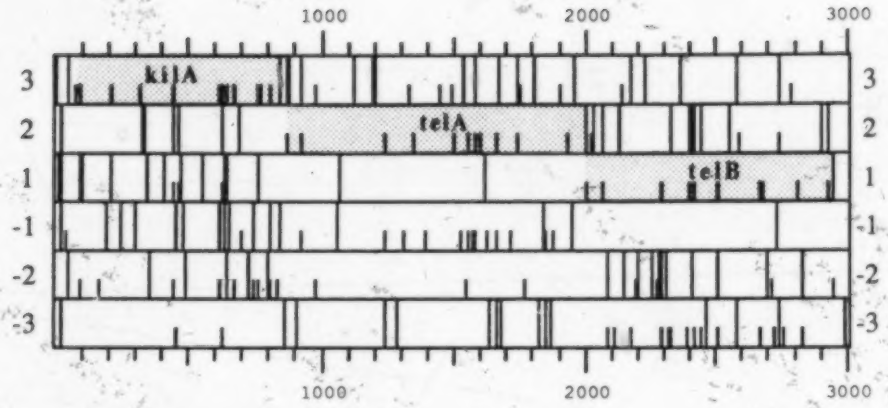


Figure 23. Open reading frames encoded by the *kilA*-Te^R region of RK2.

The positions in the DNA sequence (Figure 22) are indicated above and below in thousands of nucleotides. Possible translation-initiation codons (ATG) and termination codons (TAA, TAG, TGA) are indicated by short and full vertical lines, respectively, in the three reading frames on both the coding strand (1,2,3) and the opposite strand (-1,-2,-3). The three largest ORFs on the coding strand are shaded and marked *kilA*, *telA*, and *telB*.

is reminiscent of the signal sequences of many exported proteins (von Heijne, 1985a).

The G + C content of this region is 64 % which is close to that of other regions in RP4 (60 %; Holloway and Richmond, 1973) and in *Pseudomonas* (66 %; Marmur and Doty, 1962), and there is a corresponding preference for G or C in the third position of the codons in *kilA*, *telA*, and *telB*.

The codon adaptive index (CAI) is a measure of the frequency of usage of optimal versus nonoptimal codons (Sharp and Li, 1987a). Higher CAI values have been found to correlate with higher levels of gene expression. CAI values for the *kilA*, *telA*, and *telB* genes were determined using the DNA Strider program for the MacIntosh computer (Marck, 1988). The CAI for *kilA* was calculated to be 0.268. This value is within the range of CAI values for previously studied genes (Sharp and Li, 1987a). Similarly, the *telA* and *telB* genes have CAI values of 0.371 and 0.248 which fall within the CAI range. The lower CAI value for *telB* suggests a lower level of expression compared to *telA*.

There are several possible ORFs which are encoded on the opposite strand of the DNA (Figure 23). One ORF extends from nt 2450 to nt 1869. This ORF could potentially code for a 20.5 kDa protein. The CAI value for this ORF is 0.192, which is lower than that of even poorly expressed genes and is close to the CAI value of 0.17 that would be obtained for a gene with no codon bias (Sharp and Li, 1987b). Similarly, two other potential ORFs are located between nt 626 and nt

21, and between nt 1719 and nt 1060 on the sequence. These also have low CAI values of 0.208 and 0.099, respectively. One ORF with a CAI value of 0.295 was found on the opposite strand, extending from nt 1768 to 794. This ORF could potentially encode a 35 kDa protein. There is no evidence, however, that any of these ORFs are transcribed or translated. No active *lacZ* transcriptional or translational fusions were obtained in which mini-Mu was inserted in this orientation. In addition, no sequences similar to the Shine-Dalgarno sequence or consensus promoter sequences were found upstream of these ORFs.

Shortly after the end of *telB* is a G-C rich inverted repeat, nt 2963-2974, and 2978-2989, in which 11 out of 12 residues in each half match. A long T-rich region (nt 2984-2995) overlaps the second half of the inverted repeat. The probable stem-loop secondary structure of the mRNA complementary to this region is shown in Figure 24. The free energy of the stem was calculated to be approximately -22 kcal/mole using the Microgenie computer program. This structure strongly resembles other rho-independent terminators of transcription (Rosenberg and Court, 1979). This corresponds to the terminator previously identified upstream of the *korA* promoter (Thomas and Smith, 1986).

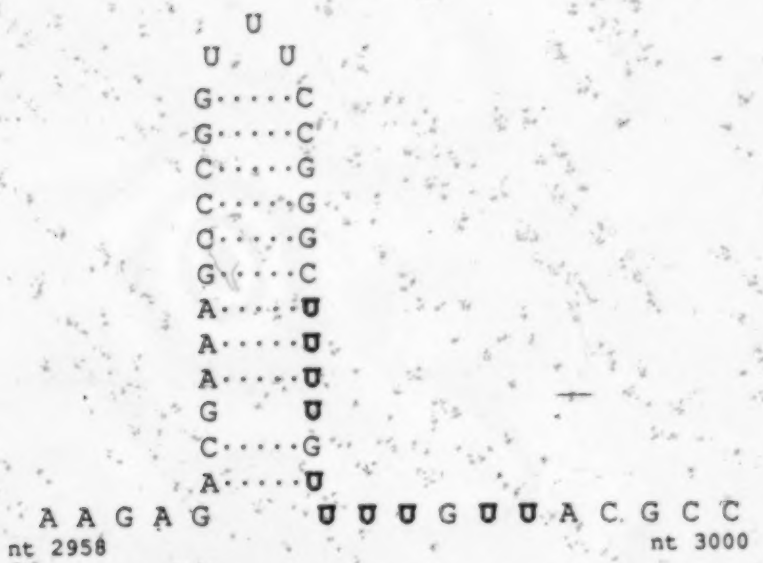
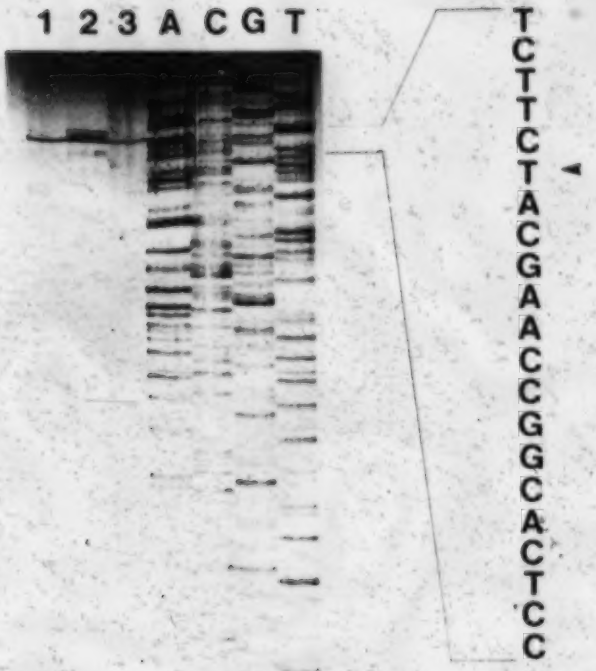


Figure 24. Transcriptional Terminator of the *tel* Genes. The probable secondary structure of the mRNA complementary to the end of the *telB* gene (nt 2958 to nt 3000) is shown. Dots represent potential hydrogen bonds between nucleotide pairs. U residues in the U-rich region at the end are indicated in bold letters.

3.13 Identification of transcription initiation sites in the IncPα Te^R region.

RNA primer extension was used to determine the start of transcription of the Te^R genes (Figure 25, 26). An oligonucleotide, 'primer 1' (5'-GTCGAAGGCGACGATAG-3'), was synthesized which was complementary to nt 903 to 919 on the sequence, near the 5' end of the *telA* gene (Figure 22). The enzyme AMV reverse transcriptase was used to extend from the oligonucleotide primer to the 5' end of the mRNA transcript. A start site was found which was identical in three bacterial strains carrying plasmid pDT1558 or RP4Te^R or RP4Te^S, and which coincided approximately with residue 321 on the sequence ("mRNA-1"; Figure 25). The exact site could not be determined due to the poor resolution of the sequencing gels in this region, approximately 600 nt from the primer. However, no similarity to the -35 and -10 promoter consensus sequences could be seen in this part of the sequence. In addition, one insertion of Tn*1000* (pDT1558-5) and several insertions of mini-Mu were isolated which were located upstream of this site, and which eliminated or sharply reduced the level of Te^R. This suggested that the actual promoter for *telA* and *telB* was close to or identical to the *kilA* promoter. Therefore, a second oligonucleotide, 'primer 2' (5'-GCGTATTGAGCATGTC-3'), was synthesized which was complementary to a region within the *kilA* gene but downstream of the first mRNA 'start' site. RNA primer extension using



mRNA-1 Primer 1

320 330 340 870 910 920

TTCGAGAAGATGCTTGGCCGTGAGGTTGA..*kilA*..ATGAAC.*telA*..CCTATCGTCGCCTTCGACATG

PheGluLysMetLeuGlyArgGluValGl.....MetAsn.....ProIleValAlaPheAspMet

Figure 25. RNA primer extension from within the *telA* gene. An oligonucleotide was synthesized which was complementary to a sequence within the *telA* gene (primer 1 in Figure 22). This was annealed to mRNA from *E. coli* carrying the plasmid pDT1558 (Lane 1), RP4Te^{R} (2), or RP4Te^{S} (3) and extension along the RNA was carried out using reverse transcriptase. The nucleotide sequence and end-products of primer extension are indicated to the right of the corresponding bands on the autoradiogram of the sequencing gel.

Figure 26. RNA primer extension from within the *kilA* gene. An oligonucleotide was synthesized which was complementary to a sequence within the *kilA* gene (primer 2 in Figure 22). This was annealed to mRNA from pDT1558 (Lane 1), RP4Te^{R} (2), or RP4Te^{S} (3), and extension along the RNA was carried out using reverse transcriptase. The nucleotide sequence and end-products of primer extension are indicated to the right of the corresponding bands on the autoradiogram of the sequencing gel.

this oligonucleotide was performed (Figure 26) and identified nt 38 in the sequence as the start site of transcription ("mRNA-2" in Figure 22). This residue is located 7 nt downstream of the -10 region, in the region where transcriptional initiation would be expected. The sequence (CAT) at this transcription initiation site is identical to that most commonly found at this position (Hawley and McLure, 1983).

3.14 Analysis of the predicted amino acid sequences of KilA, TelA, and TelB.

The amino acid sequences of KilA, TelA, and TelB were analyzed using the algorithm of Kyte and Doolittle (1982) (Figure 27). In this method, the hydrophobicity at each position was estimated by averaging the hydrophobicity values of each of the amino acids within a surrounding window of 11 amino acids. These calculated values were then plotted on a graph versus the aa residue number. Both KilA and TelA appear predominantly hydrophilic, with only a few regions in the amino acid sequence extending above the zero line. In contrast, 4 hydrophobic domains of about 20 amino acid residues in length are evident in the hydrophobicity plot of TelB. These domains may anchor TelB in the bacterial cytoplasmic membrane. This location for TelB is supported by the construction of active alkaline phosphatase fusions with this protein using the transposon Tn*phoA*. The point of

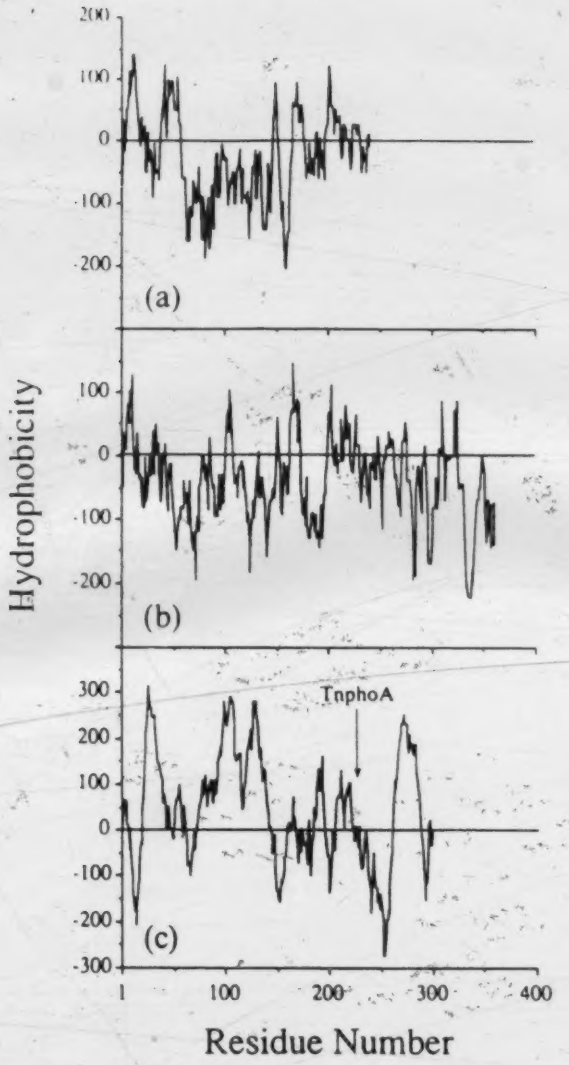


Figure 27. Hydrophobicity plot analysis of the KilA, TelA, and TelB proteins.

The deduced amino acid sequences of *kilA* (a), *telA* (b), and *telB* (c), shown in Figure 22 were used to predict the hydrophobicity plots by the algorithms of Kyte and Doolittle (1982). The positive and negative values indicate hydrophobicity and hydrophilicity, respectively. The point of insertion of Tn*phoA* in TelB is shown by an arrow in (c).

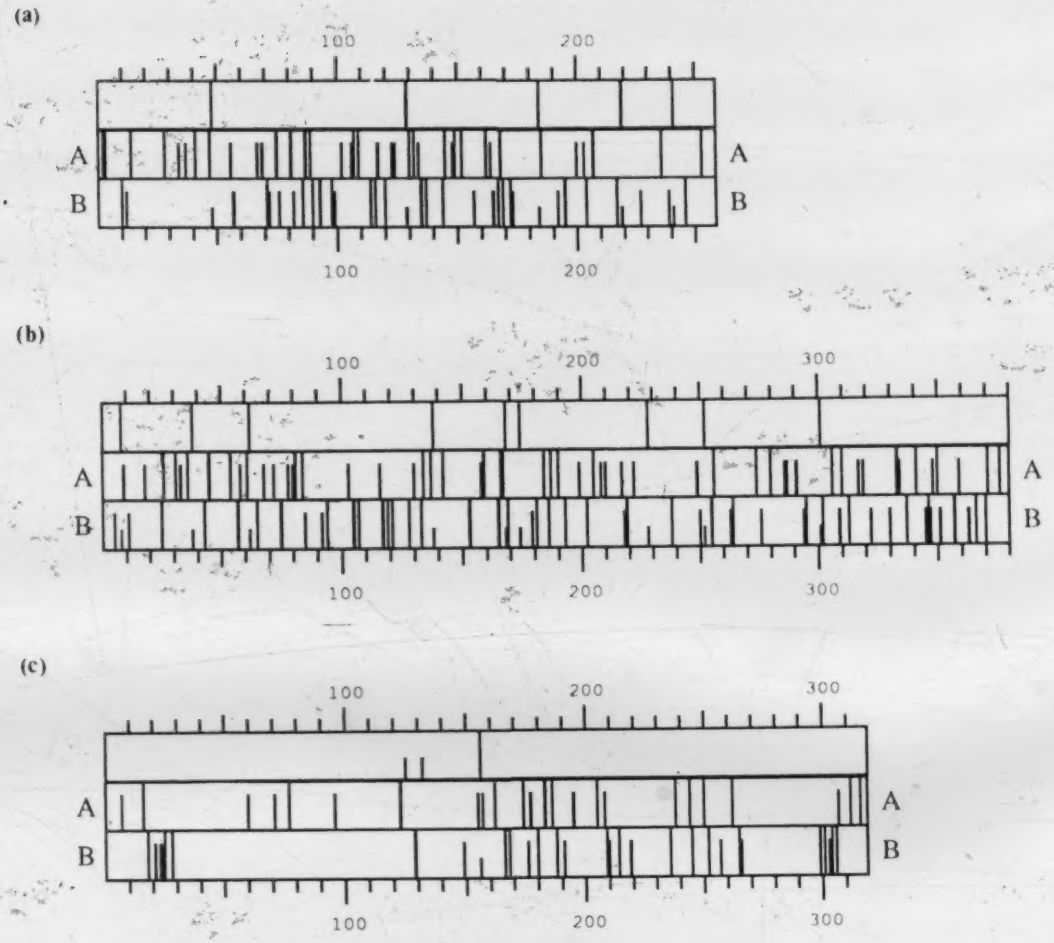


Figure 28. Locations of Acidic, Basic, Cys and His residues in KilA, TelA, and TelB.

The locations of various residues in the amino acid sequences of KilA (a), TelA (b), and TelB (c) are indicated by vertical lines in the graphs. Cysteine residues and histidine residues are indicated in the first row by short and full vertical lines, respectively. Acidic (Glu, full line; Asp, short line) and basic residues (Arg, full; Lys, intermediate; His, short line) in the sequences are indicated in the rows marked A and B, respectively.

insertion of Tn*phoA* was determined by DNA sequencing and located within amino acid residue 228 (Figure 22). This is within a relatively hydrophilic portion of the polypeptide (Figure 27). This part of the protein is predicted to be located in the periplasm since alkaline phosphatase is only active if it is exposed to the periplasmic environment (Manoil, 1990).

These locations for KilA, TelA and TelB are supported by analyses of the deduced aa sequences. KilA has 14.8 % (by number) acidic residues (Asp, Glu) and 14.4 % basic residues (His, Lys, Arg), which are spread throughout the protein (Table 10; Figure 28). Since neither acidic nor basic residues predominate, this protein is probably close to neutral overall. The relatively hydrophobic amino acids Ile, Phe, Val, Leu, Met, Ala, and Thr (IFVLMAT) make up 48 % of the polypeptide. Within the sequence of KilA there are no Cys residues and there are 5 His residues which are scattered throughout the protein (Figure 28). These residues are often found in active sites of proteins.

TelA appears to be a slightly basic polypeptide, having 14.3 % acidic and 15.3 % basic (including His) residues which are spread throughout the protein (Table 11; Figure 28). It has approximately 47 % hydrophobic residues (IFVLMAT). No Cys residues are found in this polypeptide, however there are many His residues found throughout which may be important in its function.

Table 10. Amino Acid Composition of KilA.

257 Amino Acids MW : 28391 Dalton

			n	n(%)	MW	MW(%)
A	ala	alanine	39	15.2	2770	9.8
C	cys	cysteine	-	-	-	-
D	asp	aspartic acid	15	5.8	1725	6.1
E	glu	glutamic acid	23	8.9	2967	10.5
F	phe	phenylalanine	6	2.3	882	3.1
G	gly	glycine	12	4.7	684	2.4
H	his	histidine	5	1.9	685	2.4
I	ile	isoleucine	9	3.5	1017	3.6
K	lys	lysine	13	5.1	1665	5.9
L	leu	leucine	28	10.9	3166	11.2
M	met	methionine	12	4.7	1572	5.5
N	asn	asparagine	9	3.5	1026	3.6
P	pro	proline	8	3.1	776	2.7
Q	gln	glutamine	13	5.1	1664	5.9
R	arg	arginine	19	7.4	2965	10.4
S	ser	serine	12	4.7	1044	3.7
T	thr	threonine	12	4.7	1212	4.3
V	val	valine	17	6.6	1684	5.9
W	trp	tryptophan	2	0.8	372	1.3
X	---	unknown	-	-		
Y	tyr	tyrosine	3	1.2	489	1.7
Z	---	STOP	-	-		

[a]The number and percentages (by number and by molecular weight) of each amino acid in KilA were determined from the amino acid sequence deduced from the nucleotide sequence (Figure 22) using the MacIntosh computer program DNA Strider (Marck, 1988).

Table 11. Amino Acid Composition of TelA.

378 Amino Acids MW : 42130 Dalton

			n	n(%)	MW	MW(%)
A	ala	alanine	41	10.8	2912	6.9
C	cys	cysteine	-	-	-	-
D	asp	aspartic acid	26	6.9	2990	7.1
E	glu	glutamic acid	28	7.4	3613	8.6
F	phe	phenylalanine	8	2.1	1176	2.8
G	gly	glycine	17	4.5	969	2.3
H	his	histidine	9	2.4	1233	2.9
I	ile	isoleucine	19	5.0	2148	5.1
K	lys	lysine	17	4.5	2177	5.2
L	leu	leucine	40	10.6	4523	10.7
M	met	methionine	11	2.9	1441	3.4
N	asn	asparagine	15	4.0	1710	4.1
P	pro	proline	11	2.9	1067	2.5
Q	gln	glutamine	29	7.7	3713	8.8
R	arg	arginine	32	8.5	4995	11.9
S	ser	serine	16	4.2	1392	3.3
T	thr	threonine	25	6.6	2526	6.0
V	val	valine	32	8.5	3170	7.5
W	trp	tryptophan	1	0.3	186	0.4
X	---	unknown	-	-		
Y	tyr	tyrosine	1	0.3	163	0.4
Z	---	STOP	-	-		

[a]The number and percentages (by number and by molecular weight) of each amino acid in TelA were determined from the amino acid sequence deduced from the nucleotide sequence (Figure 21) using the MacIntosh computer program DNA Strider (Marck, 1988).

The TelB polypeptide appears to be significantly different from KilA and TelA in its composition. It appears to be a more hydrophobic protein than KilA and TelA, with approximately 52 % of its residues being relatively hydrophobic (IFVLMAT; Table 12). It has only 7.6 % acidic and 9.5 % basic residues which are not spread evenly throughout the sequence (Figure 28). There are several regions which have predominantly acidic or basic residues. At the N-terminus of the polypeptide (between aa residues 18 to 28) there is a highly basic region containing 6 basic aa (Figure 22; Figure 28). This is followed by a 31 aa sequence which is uncharged and has 68 % hydrophobic IFVLMAT residues. Between aa residue 60 and 120, only acidic residues (5) are found. There are 2 long segments (of 26 and 19 aa residues) which are uncharged within this long slightly acidic region. Both of these segments have a high proportion of the hydrophobic IFVLMAT residues (81 % and 68 %, respectively). The central portion of the polypeptide is filled with both acidic and basic amino acids. Another long (33 residues) hydrophobic segment (75 % IFVLMAT) is located between residues 267 and 298. At the C-terminus, there is another highly basic region between residues 299 and 306 containing 5 positively charged residues. In the last 11 residues of the protein, 307 to 316, there are only acidic residues (3).

The algorithms of Eisenberg et al., (1984b) and Kyte and Doolittle (1982) were used to predict regions in TelB which

Table 12. Amino Acid Composition of TelB.

317 Amino Acids MW : 32375 Dalton

			n	n(%)	MW	MW(%)
A	ala	alanine	57	18.0	4049	12.5
C	cys	cysteine	2	0.6	206	0.6
D	asp	aspartic acid	10	3.2	1150	3.6
E	glu	glutamic acid	14	4.4	1806	5.6
F	phe	phenylalanine	15	4.7	2206	6.8
G	gly	glycine	41	12.9	2337	7.2
H	his	histidine	1	0.3	137	0.4
I	ile	isoleucine	18	5.7	2035	6.3
K	lys	lysine	11	3.5	1409	4.4
L	leu	leucine	21	6.6	2374	7.3
M	met	methionine	11	3.5	1441	4.5
N	asn	asparagine	5	1.6	570	1.8
P	pro	proline	16	5.0	1552	4.8
Q	gln	glutamine	8	2.5	1024	3.2
R	arg	arginine	18	5.7	2809	8.7
S	ser	serine	18	5.7	1566	4.8
T	thr	threonine	12	3.8	1212	3.7
V	val	valine	31	9.8	3071	9.5
W	trp	tryptophan	4	1.3	744	2.3
X	---	unknown	-	-		
Y	tyr	tyrosine	4	1.3	652	2.0
Z	---	STOP	-	-		

[a]The number and percentages (by number and by molecular weight) of each amino acid in TelB were determined from the amino acid sequence deduced from the nucleotide sequence (Figure 21) using the MacIntosh computer program DNA Strider (Marck, 1988).

may form membrane-spanning helices (Figure 29). These methods involve averaging the hydrophobicity values for the amino acids over windows of 21 and 19 aa residues, respectively. If the mean hydrophobicity value <H> for a window of this size exceeds the threshold value (0.42 for Eisenberg; 1.6 for Kyte Doolittle), that region in the protein is considered a possible candidate for spanning the membrane.

The four hydrophobic regions already identified in the sequence of TelB had peak <H> values of 2.4, 2.7, 1.9 and 2.1 by the algorithm of Kyte and Doolittle (1982). These values are well above the Kyte-Doolittle threshold value of 1.6 for transmembrane segments.

For comparison, the algorithm of Eisenberg et al., (1984b) for the prediction of transmembrane segments was also used (Figure 29). This algorithm uses a different hydrophobicity scale for the amino acids. The same regions in TelB had mean hydrophobicity values of 0.83, 0.87, 0.59, and 0.81 by the algorithm of Eisenberg. These values are also well above the Eisenberg threshold value of 0.42 for transmembrane domains.

A possible model for the orientation of TelB in the inner membrane of the bacterium is shown in Figure 30. This is based on the prediction that the 4 long uncharged segments described above form 4 membrane-spanning domains and that the location of insertion of Tn*phoA* is a periplasmic domain.

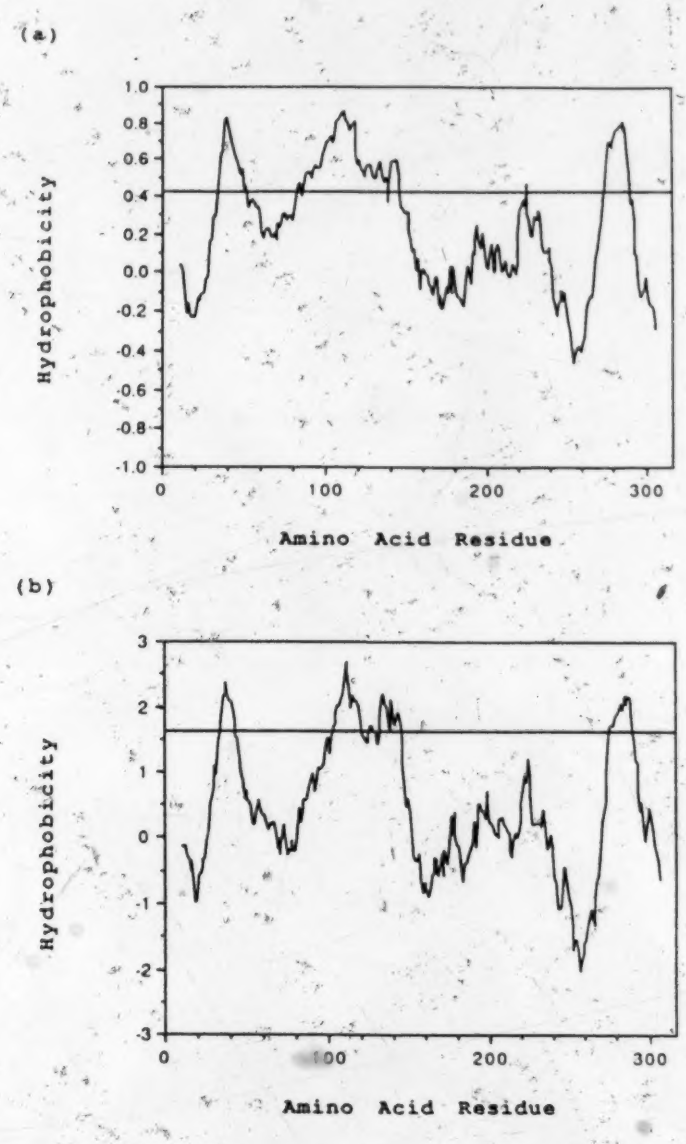


Figure 29. Prediction of membrane-spanning regions in TelB. The transmembrane segment prediction algorithms of (a) Eisenberg et al., (1984b) and (b) Kyte and Doolittle (1982) were used to determine the mean hydrophobicity values in the TelB amino acid sequence over windows of 21 and 19 amino acids, respectively. The threshold values of 0.42 (a) and 1.6 (b) are indicated by dotted horizontal lines.

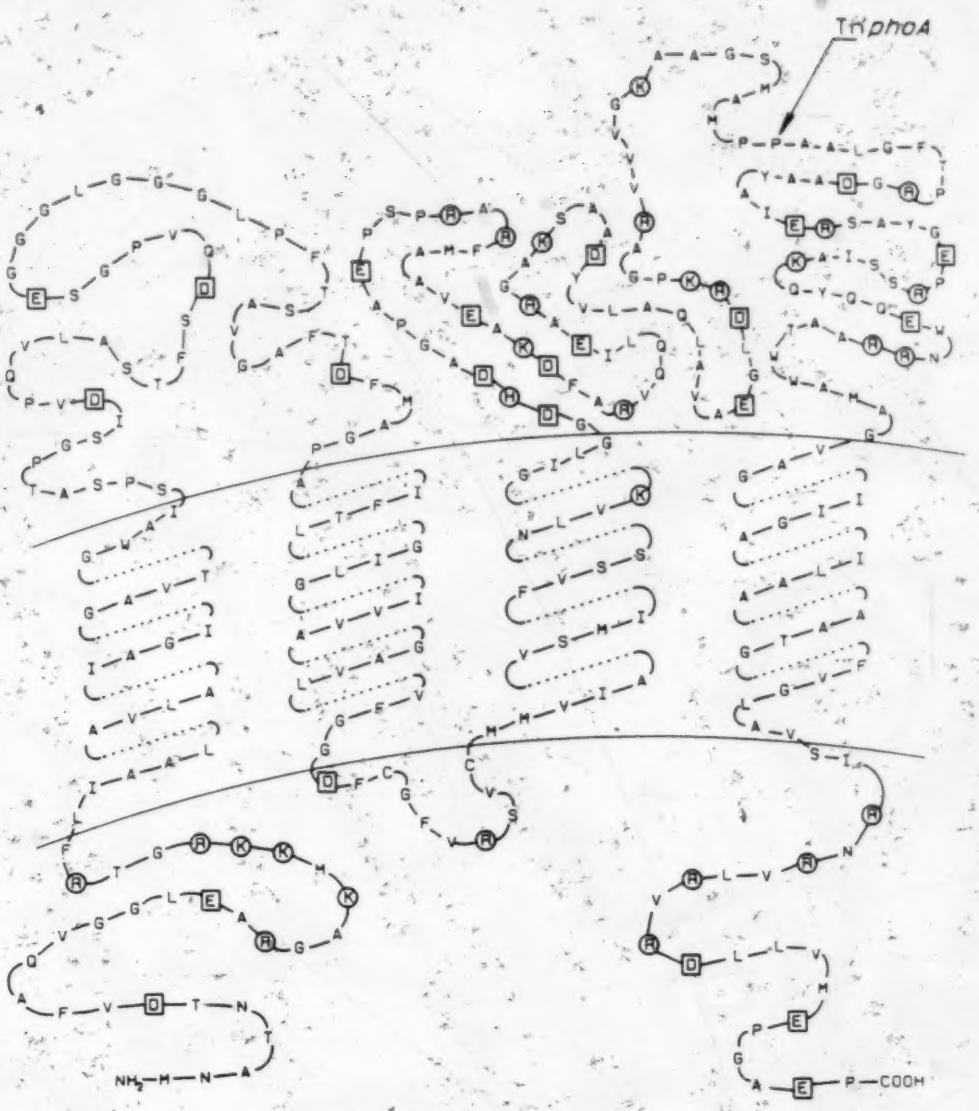


Figure 30. Model of TelB.

A possible model for the topology of the Te^R protein TelB in the inner membrane of *E. coli* is shown. Acidic and basic residues in the sequence are indicated by circles and squares, respectively. The point of insertion of Tn*phoA* in a $PhoA^+$ Te^S fusion is indicated.

The distribution of positively charged residues has been used to predict cytoplasmic and periplasmic domains (von Heijne, 1986). In the proposed model of TelB, the first cytoplasmic domain contains 6 positively charged residues (including His) and only 2 negatively charged aa. The second and third putative cytoplasmic domains have ratios of positive to negative aa of 1:1 and 5:3, respectively. This predominance of basic amino acids in the cytoplasmic domains is consistent with previous studies of inner membrane proteins. The first periplasmic domain has four negatively charged residues and no positively charged aa. However, the second putative periplasmic domain, containing approximately 122 aa, has 17 basic aa and only 14 acidic residues. This does not fit as well with previous observations that fewer positive residues are found in periplasmic domains. However, some of this region of the polypeptide is expected to be in the periplasm since an active ($PhoA^+$) fusion to alkaline phosphatase was obtained in this region.

Proline residues have been found to be more common in periplasmic loops versus cytoplasmic loops and in cytoplasmic-pointing versus periplasmic-pointing membrane spanning regions (von Heijne, 1986). In TelB, there are 6 Pro residues in the first periplasmic loop and 8 in the second periplasmic loop (Figure 30). In contrast only 2 Pro residues are found in the regions which were placed in the cytoplasm. No Pro residues were located in the membrane-spanning domains. Thus, in its distribution of Pro residues,

this model is consistent with the observations of von Heijne (1986).

3.15 Comparison of the DNA sequences of RK2Te^R and RK2Te^S.

The DNA sequence of the corresponding region of a tellurite-sensitive isolate of RK2 was determined by Chris Thomas and John Ibbotson (University of Birmingham, U. K.; Walter, Thomas, Ibbotson, and Taylor, submitted for publication). The difference between RK2Te^R and RK2Te^S was investigated by comparing their nucleotide sequences in the Te^R region (Figure 21, Table 13). Over the 3 kb region of DNA compared, only 4 differences in the nucleotide sequence were discovered. Two of these differences were frame-shift mutations within the *telA* and *telB* genes at nt positions 1282 and 2522, respectively. These differences would result in incorrect amino acids in the carboxyl-termini and premature termination of both the TelA and TelB proteins encoded by RK2 at nt positions 1611 and 2548, respectively (Figure 22).

Another difference between RK2 and RK2Te^R is a missense mutation (A to T) at nt residue 2371 which would result in a Ser to Cys transition at aa residue 125 in TelB. Another Cys residue is located 7 aa away. This Cys..Cys region resembles metal and cofactor binding sites in proteins such as mercuric reductase and glutathione reductase (described below), thus it may be important in the function of TelB.

Table 13. Differences in the nucleotide sequence of the Te^R region of RK2 and $RK2Te^R$.

Position (gene)	RK2	$RK2Te^R$	Type of mutation
1282 (*telA*)	-	G	frame-shift
2371 (*telB*)	A	T	missense (Ser to Cys)
2522 (*telB*)	-	C	frame-shift
2966 (within transcriptional terminator)	-	A	alters inverted repeat

The fourth difference is an A residue found at position 2966 in RK2Te^R which is not found in RK2. This is within the transcriptional terminator (Figure 24) and results in improved symmetry of the inverted repeat in RK2Te^R.

3.16 Comparison of the IncPα Te^R sequence with other sequences.

The nucleotide and amino acid sequences from the IncPα Te^R region were compared with those from the IncHI2 Te^R region of pMER610 (Jobling and Ritchie, 1988). No significant homology could be found. One of the IncHI2 Te^R ORFs (ORF3) is predicted to encode a protein of 38.2 kDa which appears to be located in the membrane based on hydrophobicity plot analysis. This is close to the size of the putative membrane protein TelB. The sequence of the ORF3 polypeptide contains 55 % hydrophobic residues (IFVLMAT), 11 % acidic residues and 8.7 % basic residues. Thus, in contrast to TelB, ORF3 appears to be slightly acidic. In addition, the protein has only 1 Cys residue and 11 His residues. It is predicted to have 9 membrane-spanning domains (Jobling and Ritchie, 1988). The <H> values for each of these putative transmembrane domains are well above the threshold values for both the Kyte and Doolittle (1982) and Eisenberg et al., (1984b) algorithms.

Current DNA and protein data banks were searched for proteins having homology to KilA, TelA or TelB. Small

regions of similarity with other proteins could be found, however there was no overall homology (Figure 31). A short (66 aa) region in KilA was found to have 40.6 % homology (including the conservative changes listed in Appendix 3) with a region in adenylate kinase from *Paracoccus denitrificans* (Edinburgh DAP (Prosrch) Database).

Several proteins were found to have small regions of similarity with hydrophobic segments of TelB (Figure 31). A highly hydrophobic region of Colicin A from *Citrobacter freundii* (Morlon et al., 1983) had 71 % homology with a 31 aa segment of TelB which included one of the putative membrane-spanning domains (Figure 30; Figure 31). 49 % homology was found between the N-terminal sequences of TelB and the protein Aleurain, a barley thiol protease (Rogers et al., 1985; Figure 31). This part of TelB is relatively hydrophobic and includes one of the putative membrane-spanning regions (Figure 30).

A relatively long region (82 aa) of TelB was found to have 41.4 % homology with a region of the proline carrier protein, PutP, of *Escherichia coli* which contains two potential membrane-spanning domains (Nakao et al., 1987; Figure 31). The homologous region in TelB also contains two putative membrane-spanning domains as well as the two closely spaced Cys residues which may be located in the cytoplasm and a region thought to be located in the periplasm (Figure 30).

The sequence was examined for the presence of homology with nucleotide-binding regions. A consensus sequence,

Figure 31. Sequence similarities in the IncPα Te^R region.

The regions of similarity between either KilA or TelB and proteins identified during searches against the current DNA and proteins sequence banks are indicated.

(A) Similarity between KilA and adenylate kinase from *Paracoccus denitrificans*. (B) Comparison of TelB and Colicin A from *Citrobacter freundii* (Morlon et al., 1983). (C) Comparison of TelB and Aleurain (Rogers et al., 1985). (D) Comparison of TelB and the proline carrier protein (*putP*) from *Escherichia coli* (Nakao et al., 1987).

Identity between amino acids in different proteins is indicated by '*'. Conservative amino acid changes (Appendix 3) are indicated by '|'. Spaces inserted in the sequence to optimize the alignment are indicated by '.'. The start and end positions in the amino acid sequences of the proteins are indicated at the left and right of each line. Percent similarity was determined by adding up the number of identical and conserved aa residues and dividing by the total length (including spaces) of the compared region in TelB.

(A) KilA vs Adenylate kinase from *Paracoccus denitrificans* (40.6 % homology)

```
LTPQQQQLVTQMKVA.PIPKQLLQRIPNIPPN.INTWQQVT.ALA
*       *****||      |* *        |  * * * **
LMASHEMSVTQMKLTRAQAVDMLDRFSETASVLVPVWRQHTLALI

QQKLLTPQDMEAAKEVYKIHQQLL
  * | *  *  * *  * ** *|
TTKNMNP.AM.VA.EA.KAHQALM
```

(B) TelB vs Colicin A from *Citrobacter freundii*.(71 % homology)

```
88        GTSAVGAFTDFMAGPAIFTLGILGIVVAGAV     118
           ||||**||  |  *** ||** **||*  *
542       FSATLGAYALSLGVPAI AVGIAGILLAAVV     571
```

(C) TelB vs Aleurain (49 % homology)

```
28  RFLIAALAVLAIAGIGAVTGWAISPS.ATPGSID.VPQVLASTFSDQVPGSDG  78
    * *| ****** * * **   * * * *    *  *    ***    * *| *
5   RVLLLALAVLATAAV.AV...ASSSSFADSNPIRPVTDRAASTLESAVLGALG  53
```

(D) TelB vs the proline carrier protein from *Escherichia coli* (41.4 % homology)

```
90        AVGAFTDFM.AGPAIFTLGILGIVVAGAVLVFGGEFCGFVRSVCMMV.I
          **   ** | *  **|* **  *|     * ** * *   *    *
182       AVSW.TDTVQASLMIFAL.ILTPVIVIIS.V.GG.F.GDSLEVIKQKSI

156       A.VSMIFVSSNLVKGI.LGGDHDAGPAEPSPRARFMAA   172
          * *|   * *  * * *        *   ******
244       ENVDMLKGL.NFVAIISLMGWGLGYFGQPHILARFMAA   261
```

G....GKS/T has been discovered in proteins of both prokaryotic and eukaryotic origin that bind ATP (Higgins et al., 1986). This sequence was not found in KilA, TelA or TelB. Similarly no homology was found with DNA binding proteins which have a consensus α-helix/β-turn/α-helix region containing the sequence **A...G.....I/L/V** (Pabo and Sauer, 1984; Dodd and Egan, 1987).

Many proteins, including mercuric reductase (MerA), the periplasmic mercury binding protein (MerP), glutathione reductase (Gor), and the cadmium resistance protein (CadA), have homology in regions thought to be involved in cation binding (Perham, 1987; Nucifora et al., 1989a). This region includes a pair of Cys residues (Figure 32). TelB also contains two closely spaced Cys residues which may be located in the cytoplasm (Figure 30). This region of TelB was compared with the cation binding regions of MerA, MerP, CadA, and Gor (Figure 32a). There was only a small amount of homology between the closely related regions of CadA, MerA and MerP and the Cys...Cys region of TelB. In TelB, the two Cys residues are separated by 6 aa whereas in CadA, MerA and MerP, the two Cys are separated by only 2 residues. Another region of MerA containing 2 Cys which might be involved in binding mercury ions or cofactors, has much more homology to TelB (Figure 32b). There is less difference in the distance separating the 2 Cys residues and several other amino acids in the region are identical or conserved. This Cys..Cys region in TelB is a possible site for binding to metals or

Figure 32. Comparison of TelB to cation binding proteins.

(A) Sequence homology between regions of CadA, MerA, MerP, and Gor (Nucifora et al., 1989; Perham, 1987).

(B) Comparison of TelB to regions of CadA, MerA, MerP, and Gor.

Identity between amino acids in different proteins is indicated by '*'. Conservative amino acid changes (Appendix 3) are indicated by '|'. Horizontal lines above amino acid residues indicate those which increase the spacing between the two Cys residues. The start and end positions in the amino acid sequences of the proteins are indicated at the left and right of each line.

(A) Hypothesized cation (or cofactor) binding regions

```
Consensus                 |  * **  *        | *| **
CadA            18        VQGFTCANCAGKFEKNVKKIPGV          40
MerA             6        ITGMTCDSCAVHVKDALEKVPGV          28
MerP            28        VPGMTCAACPITVKKALSKVEGV          50

                            * **    *
Gor             37        ELGGTCVNVGCVPKKVMWHAAQIRE        61

                               *      *        |  |  |
Gor            384        THRQPCRMKLVCVGSEEKIVGIHGIGF      410

                            * **     *          |
MerA           130        TIGGTCVNVGCVPSKIMIRAAHIAH        154
```

(B) Comparison of TelB with cation binding regions

```
CadA            18        VQGFTC    ANCAGKFEKNVKKIPGV      40
                            *  *_____| *        |  |
TelB           120        FGGEFCGFVRSVCMMVIAVSMIFVSSN      146

MerA             6        ITGMTCD   S CAVHVKDALEKVPGV      28
                            *  * ___*_* | |  || *
TelB           120        FGGEFCGFVRSVCMMVIAVSMIFVSSN      146

MerA           130        TIGGTC  VNVGCVPSKIMIRAAHIAH     154
                            *  *__*   *|    |      |
TelB           120        FGGEFCGFVRSVCMMVIAVSMIFVSSN      146

MerP            28        VPGMTC    AACPITVKKALSKVEGV      50
                             *_____| * | |  || *
TelB           120        FGGEFCGFVRSVCMMVIAVSMIFVSSN      146

Gor             37        ELGGTC  VNVGCVPKKVMWHAAQIRE      61
                            *  *  *   *|    |
TelB           120        FGGEFCGFVRSVCMMVIAVSMIFVSSN      146

Gor            384        THRQPCR MKLVCVGSEEKIVGIHGIGF    410
                             * _|| **|       |
TelB           120        FGGEFCGFVRSVCMMVIAVSMIFVSSN      146
```

reducing equivalents. A comparison of the Cys..Cys regions in TelB and Gor also shows several synonymous or identical amino acids.

3.17 DNA sequencing analysis of the IncHII Te^R genes.

The DNA sequence of the IncHII Te^R region was determined by subcloning restriction fragments from pDT1364 or its Tn*1000* insertion mutants into M13mp18 or M13mp19 for single-stranded sequencing (Figure 33). Double-stranded sequencing was carried out with the Te^R subclone, pDT1947 (see section 3.21), or the Tn*1000* or Tn*phoA* insertion mutants using specially designed oligonucleotide primers. Both coding and non-coding strands were sequenced.

Potential open reading frames (ORFs) were identified by localization of probable initiation codons (ATG) and termination codons (TAA, TAG, TGA) (Figure 34). Two large ORFS were identified (Figure 35). These were named *tehA* and *tehB* to distinguish IncHII plasmid Te^R genes from those on RK2.

The first ORF, *tehA*, extends from nt 133 to 1122 in the sequence (Figure 34). It can potentially code for a protein of 330 amino acids with a molecular mass of 35,908 daltons. The start codon for this ORF is preceded by a possible ribosome binding site (GAG) 7 nt upstream. Another methionine codon is located 4 codons upstream of this start

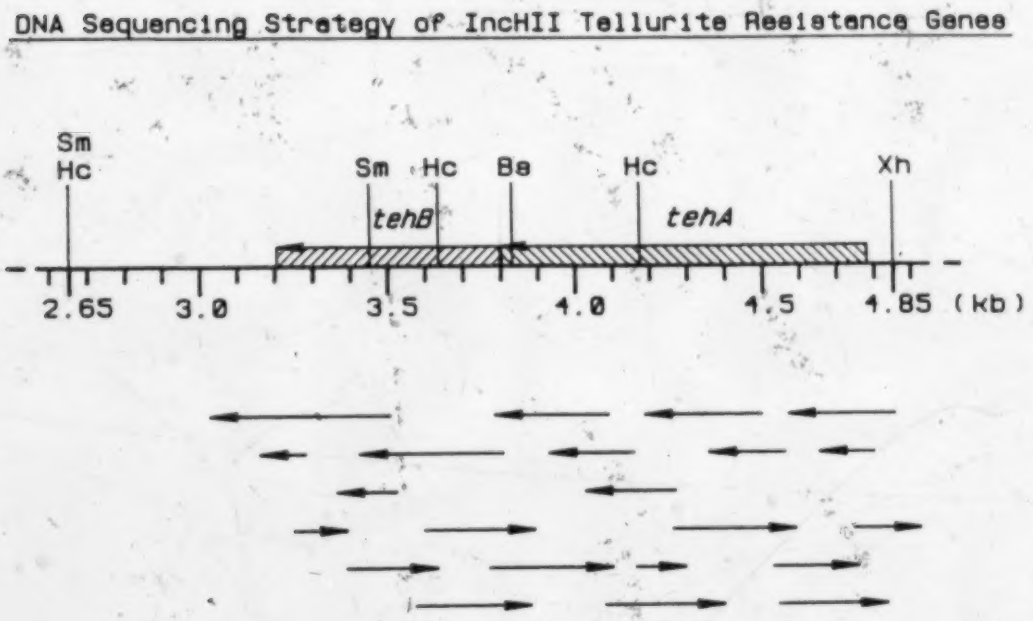


Figure 33. DNA sequencing strategy for the IncHII Te^R region.

The restriction map of the Te^R region from pHH1508a is shown. The direction and extent of sequencing in individual subclones is indicated by arrows below the map. The two largest open reading frames are indicated on the map by cross-hatching.

site; however, no sequences resembling the Shine-Dalgarno ribosome binding site could be found upstream of this site.

An A-T rich region (TAAAAT), located from nt residue 82 to 87 on the sequence, was found upstream of the *tehA* ORF. This has 5 out of 6 residues the same as the *E. coli* -10 consensus sequence (TATAAT). There is also some similarity to less well-conserved positions in the region upstream of this hexamer (Rosenberg and Court, 1979). A hexanucleotide (CTGGCT) with 3 out of 6 residues the same as the -35 consensus sequence (TTGACA) was located 18 nt upstream of the proposed -10 region.

The point of insertion of Tn*phoA* into the *tehA* gene was found to be immediately after nt residue 247 within aa residue 39. It is inserted in the correct reading frame for production of a TehA::PhoA fusion protein (Figure 18). The DNA sequence upstream of the insertion site also has some similarity to hot-spot sites for transposition of Tn*phoA* identified by Gött and Boos (1988).

The second ORF, *tehB*, extends from nt residues 1122 to 1712 (Figure 34). It can code for a protein of 198 aa with a predicted molecular mass of 22,516 daltons. One nt of the start codon for *tehB* overlaps with the stop codon for *tehA*. There is no similarity to consensus promoter sequences upstream of the *tehB* gene, suggesting that *tehA* and *tehB* are transcribed as a single unit. However, a possible RBS (AGAGGA) is located 6 nt upstream of the ATG start codon for

Figure 34. Nucleotide and predicted amino acid sequences of the Te^R genes from pHH1508a.

The nucleotide sequence of the coding strand of the Te^R region from pHH1508a is shown. The deduced amino acid sequences of the two largest open reading frames, *tehA* and *tehB*, are shown underneath. Hexanucleotides corresponding to the probable transcription initiation site are indicated by "-35" and "-10". Possible ribosome binding (Shine-Dalgarno) sequences are marked "SD". mRNA start sites determined by RNA primer extension are marked with a vertical line and "mRNA". The site complementary to the oligonucleotide made for primer extension is marked "Primer". Points of insertion of Tn*phoA* and Tn*1000* in various insertion mutants are also indicated above the sequence. A region at the end of *tehB* which may result in a stem-loop structure in the mRNA is indicated by a horizontal line above the sequence. A T-rich sequence in the same region is underlined.

```
        10         20         30         40         50         60
TTGCGCATAGCGTTTTCCCACAGGTGACTT ACTATAACCGTAGCAAATTCTGCGGCTCTG
-35     Xh                 -10          mRNA
___  ___70___       80 _____ 90     |   100   |   110|        120
GCTATGCTCGAGAAAATTCCATAAAATGCA TTTCAAATATACCTTTATAAATTAAACAAA
  SD
___ 130        140        150        160        170        180
ATGAGTAAGAAGATGCAGAGCGATAAAGTG CTCAATTTGCCGGCAGGCTACTTTGGTATT
          MetGlnSerAspLysVal LeuAsnLeuProAlaGlyTyrPheGlyIle   (tehA)

       190        200        210        220        230        240
GTGTTGGGGACGATAGGGATGGGATTTGCC TGGCGCTATGCCAGCCAGGTTTGGCAGGTC
ValLeuGlyThrIleGlyMetGlyPheAla TrpArgTyrAlaSerGlnValTrpGlnVal
   TnphoA                Primer
      250      < 260      270        280        290        300
AGCCACTGGTTAGGGGATGGGCTGGTGATT CTGGCGATGATCATCTGGGGATTATTGACT
SerHisTrpLeuGlyAspGlyLeuValIle LeuAlaMetIleIleTrpGlyLeuLeuThr

       310        320        330        340        350        360
AGCGCATTTATTGCCCGACTCATACGCTTT CCGCATAGCGTGCTGGCGGAAGTTCGCCAT
SerAlaPheIleAlaArgLeuIleArgPhe ProHisSerValLeuAlaGluValArgHis

       370        380        390        400        410        420
CCAGTGCTGAGCAGTTTTGTGAGTTTGTTT CCGGCAACGACGATGCTGGTGGCGATTGGT
ProValLeuSerSerPheValSerLeuPhe ProAlaThrThrMetLeuValAlaIleGly

       430        440        450        460        470        480
TTTGTTCCGTGGTTTCGCCCACTGGCGGTG TGCCTGTTCAGTTTTGGTGTCGTGGTTCAG
PheValProTrpPheArgProLeuAlaVal CysLeuPheSerPheGlyValValValGln

       490        500        510        520        530        540
TTGGCTTATGCCGCCTGGCAAACTGCGGGA TTATGGCGCGGATCTCACCCTGAAGAAGCT
LeuAlaTyrAlaAlaTrpGlnThrAlaGly LeuTrpArgGlySerHisProGluGluAla

       550        560        570        580        590        600
ACCACGCCTGGACTGTATCTGCCGACAGTT GCCAACAACTTTATCAGCGCAATGGCCTGT
ThrThrProGlyLeuTyrLeuProThrVal AlaAsnAsnPheIleSerAlaMetAlaCys

       610        620        630        640        650        660
GGTGCGTTGGGCTACACCGACGCCGGTCTG GTGTTTTTAGGCGCAGGCGTTTTCTCATGG
GlyAlaLeuGlyTyrThrAspAlaGlyLeu ValPheLeuGlyAlaGlyValPheSerTrp

       670        680        690        700        710        720
CTAAGCCTTGAACCGGTGATCTTGCAGCGT CTGCGCAGTTCGGGAGAATTACCCACGGCA
LeuSerLeuGluProValIleLeuGlnArg LeuArgSerSerGlyGluLeuProThrAla

       730        740        750        760        770        780
CTGCGGACATCACTCGGCATTCAGCTCGCT CCTGCGCTGGTGGCTTGTAGTGCCTGGCTG
LeuArgThrSerLeuGlyIleGlnLeuAla ProAlaLeuValAlaCysSerAlaTrpLeu
```

```
                                                                    Hc
      790        800        810        820        830        840
AGCGTCAACGGCGGCGAGGGTGACACGCTG GCGAAAATGCTTTTCGGTTATGGACTGCTG
SerValAsnGlyGlyGluGlyAspThrLeu AlaLysMetLeuPheGlyTyrGlyLeuLeu
                                                  Tn1000 (pDT1364-8)
      850        860        870        880        890        900
CAACTGCTGTTTATGCTACGTCTGATGCCA TGGTATCTCTCCCAGCCATTTAATGCTTCA
GlnLeuLeuPheMetLeuArgLeuMetPro TrpTyrLeuSerGlnProPheAsnAlaSer

      910        920        930        940        950        960
TTCTGGAGTTTCTCGTTCGGCGTATCTGCA CTGGCAACCACCGGTTTGCATCTGGGGAGT
PheTrpSerPheSerPheGlyValSerAla LeuAlaThrThrGlyLeuHisLeuGlySer

      970        980        990       1000       1010       1020
GGCAGCGATAATGGATTTTTCCATACGCTG GCGGTGCCGCTGTTTATCTTTACCAATTTT
GlySerAspAsnGlyPhePheHisThrLeu AlaValProLeuPheIlePheThrAsnPhe

     1030       1040       1050       1060       1070       1080
ATTATTGCAATACTGCTCATCCGTACTTTT GCGCTTCTGATGCAGGGAAAATTGTTAGTC
IleIleAlaIleLeuLeuIleArgThrPhe AlaLeuLeuMetGlnGlyLysLeuLeuVal
          Bs                                SD
     1090       1100       1110       1120       1130       1140
AGAACCGAGCGCGCCGTTTTAATGAAAGCA GAGGACAAAGAATGATCATTCGTGAGGAAA
ArgThrGluArgAlaValLeuMetLysAla GluAspLysGlu
                                                MetIleIleArgAspGluA  (tehB)
                                                  Tn1000 (pDT1364-6)
     1150       1160       1170       1180       1190       1200
ACTATTTTACTGATAAATATGAATTAACCC GCACACACTCTGAAGTACTGGAAGCGGTGA
snTyrPheThrAspLysTyrGluLeuThrA rgThrHisSerGluValLeuGluAlaValL

     1210       1220       1230       1240       1250       1260
AAGTGGTTAAACCGGGTAAAACGCTGGATC TGGGCTGTGGCAATGGTCGTAACAGTCTTT
ysValValLysProGlyLysThrLeuAspL euGlyCysGlyAsnGlyArgAsnSerLeuT
                                    Hc
     1270       1280       1290       1300       1310       1320
ACCTGGCAGCCAATGGTTATGATGTTGACG CATGGGATAAAAATGCCATGAGTATCGCCA
yrLeuAlaAlaAsnGlyTyrAspValAspA laTrpAspLysAsnAlaMetSerIleAlaA
                                                  Tn1000 (pDT1364-1)
     1330       1340       1350       1360       1370       1380
ACGTCGAGCGCATTAAATCCATTGAAAATC TGGATAATTTACACACCCGAGTCGTTGATC
snValGluArgIleLysSerIleGluAsnL euAspAsnLeuHisThrArgValValAspL

     1390       1400       1410       1420       1430       1440
TGAATAACCTCACATTTGATAGACAGTACG ATTTTATTCTTTCGACTGTGGTGCTGATGT
euAsnAsnLeuThrPheAspArgGlnTyrA spPheIleLeuSerThrValValLeuMetP
                                    Sm
     1450       1460       1470       1480       1490       1500
TCCTTGAGGCTAAAACCATCCCCGGGTTGA TTGCCAATATGCAACGTTGCACTAAACCTG
heLeuGluAlaLysThrIleProGlyLeuI leAlaAsnMetGlnArgCysThrLysProG

     1510       1520       1530       1540       1550       1560
GTGGTTACAACCTGATTGTGGCGGCGATGG ATACCGCTGATTATCCATGTACCGTCGGCT
lyGlyTyrAsnLeuIleValAlaAlaMetA spThrAlaAspTyrProCysThrValGlyP
```

```
      1570       1580       1590       1600       1610       1620
TCCCGTTTGCCTTCAAAGAGGGAGAATTAC GTCGATATTACGAAGGCTGGGAGAGGGTGA
hePro PheAlaPheLysGluGlyGluLeuA rgArgTyrTyrGluGlyTrpGluArgValL

      1630       1640       1650       1660       1670       1680
AATACAATGAAGACGTCGGCGAGCTGCACC GCACCGACGCCAACGGTAATCGTATTAAAC
ysTyrAsnGluAspValGlyGluLeuHisA rgThrAspAlaAsnGlyAsnArgIleLysL

      1690       1700       1710       1720       1730       1740
TGCGTTTCGCCACGATGCTGGCACGTAAAA AATGACCCGGTAAGCACAAAACGCGTGAAA
euArgPheAlaThrMetLeuAlaArgLysL ys

      1750       1760       1770       1780       1790       1800
ATTCCCCACGCTGAGATGATTTACTGTTCT TCTTTTCGGTAAGCATATTTTTTATCGAAG

      1810       1820       1830       1840       1850       1860
GGATGTGAAATTAATCACAGTAGTCGAAGT TTTTAGCAGCTTAACTTACTGAAATTTAAG

      1870       1880       1890       1900       1910       1920
ACTGATGATTGACTTAGCCCCTTTTTCGGC ATTGACTATGTCGTCTGAAAAGGGGCTCAA

      1930
AAATTATTTCACCAA
```

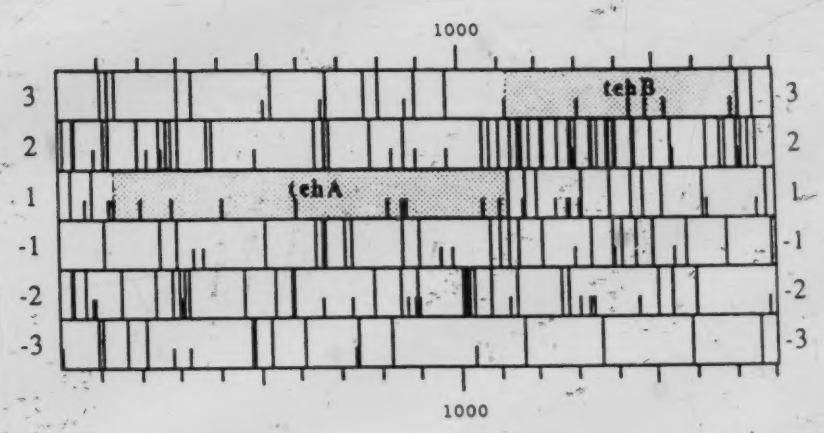


Figure 35. Open reading frames in the IncHII Te^R sequence. The positions in the DNA sequence (Figure 34) are indicated above and below in thousands of nucleotides. Possible translation-initiation codons (ATG) and termination codons (TAA, TAG, TGA) are indicated by short and full vertical lines, respectively, in the three reading frames on both the coding strand (1,2,3) and the opposite strand (-1,-2,-3). The two largest ORFs on the coding strand are shaded and marked *tehA* and *tehB*.

this ORF. No large potential ORFs were found in the opposite reading frame of the sequence (Figure 35).

The CAI values for the *tehA* and *tehB* genes were determined to be 0.251 and 0.344, respectively. These values are within the range of CAI values for previously studied genes (Sharp and Li, 1987a). The smaller value for *tehA* suggests that this gene is expressed at a lower level than the *tehB* gene.

A possible transcriptional terminator was identified downstream of the *tehB* gene. The regions between nt 1754 to 1766 and 1777 to 1788 are complementary in nine positions (out of 13 and 12 residues, respectively). The corresponding region in the mRNA could potentially form a weak stem-loop structure; however, the calculated free-energy value is close to 0 kCal/mole. A T-rich region, in which 8 out of 10 residues are T, extends from nt 1786 to 1795, overlapping the putative stem-loop structure. The possible secondary structure of the mRNA complementary to this region is shown in Figure 36. This region resembles other rho-independent transcriptional terminators (Rosenberg and Court, 1979).

The G + C content of the IncHII Te^R region sequenced was found to be 48 % which is close to the values determined for plasmids in the HI and HII groups (49-50 %; Whiteley and Taylor, 1983) and *E. coli* chromosomal DNA (50 %; Marmur and Doty, 1962). There is a corresponding percentage G + C in the third position of the codons in *tehA* and *tehB*.

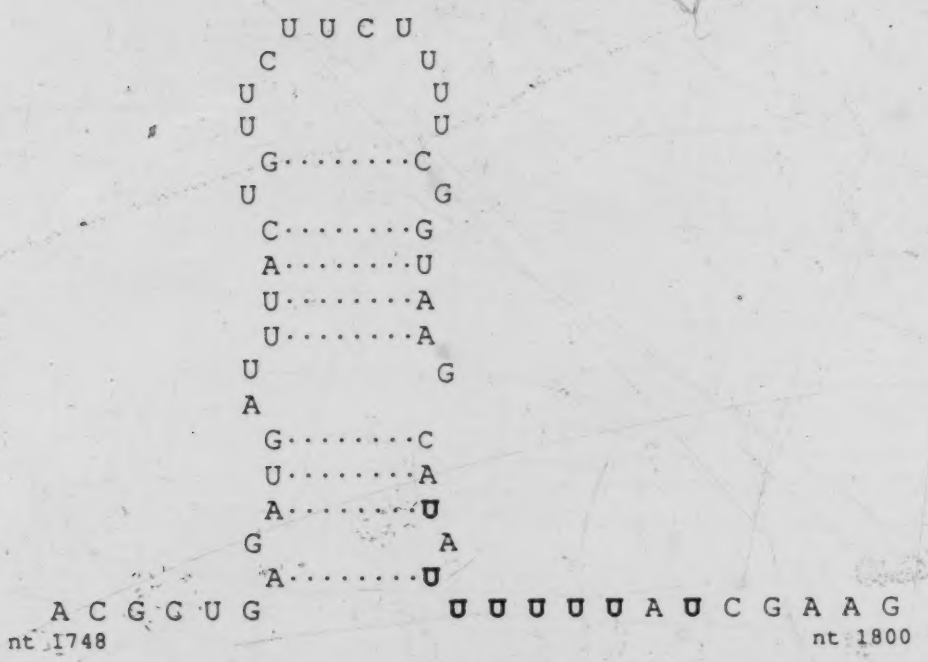


Figure 36. Transcriptional Terminator of the *teh* genes.

The probable secondary structure of the mRNA complementary to the end of the *tehB* gene (nt 1748 to nt 1800) is shown. Dots represent potential hydrogen bonds between nucleotide pairs. U residues within the U-rich region at the end are indicated in bold letters.

3.18 Identification of transcription initiation sites in the IncHII Te^R region.

RNA primer extension was carried out to confirm the start site of transcription of the IncHII Te^R genes. An oligonucleotide was synthesized which was complementary to nt residues 257 to 277 on the sequence. The enzyme AMV reverse transcriptase was used to extend the mRNA. Several potential start sites were identified (Figure 37). One of these coincided with nt residue 94 on the sequence which is located 7 nt downstream of the proposed -10 region. The other start sites identified may result from RNA polymerase initiating transcription at other weak promoter-like sequences on the DNA. Alternatively, the mRNA may have been degraded *in vivo* or *in vitro*, resulting in false start sites being identified.

3.19 Analysis of the predicted amino acid sequences of TehA and TehB.

The amino acid sequences of TehA and TehB were analyzed using the algorithms of Kyte and Doolittle (1982) using a window of 11 amino acids (Figure 38). TehA appears predominantly hydrophobic. At least eight highly hydrophobic regions of about 20 aa in length are evident. These may form membrane-spanning domains. The position of insertion of Tn*phoA* in an active ($PhoA^+$) fusion with TehA is immediately after the first of these putative membrane-spanning regions within aa residue 39. This part of the protein is predicted

Figure 37. RNA primer extension of the HII Te^R genes.

An oligonucleotide was synthesized which was complementary to a sequence within the *tehA* gene (Figure 34). This was annealed to mRNA from *E. coli* carrying the plasmid pDT1364 and extension along the RNA was carried out using reverse transcriptase. The product of RNA primer extension is shown in the lane marked RNA beside the products of sequencing reactions of pDT1364 using this primer. The nucleotide sequence and end-products of primer extension are indicated to the right of the corresponding bands on the autoradiogram of the sequencing gel.

-35 -10 mRNA

70 80 90 100 110 120

CTGGCTATGCTCGAGAAAATTCCATAAAATGCATTTCAAATATACCTTTATAAATTAAACAAA...

Primer

< 260 270 280

tehA...GGGGATGGGCTGGTGATTCTGGCGATGA...

...GlyAspGlyLeuValIleLeuAlaMetI...

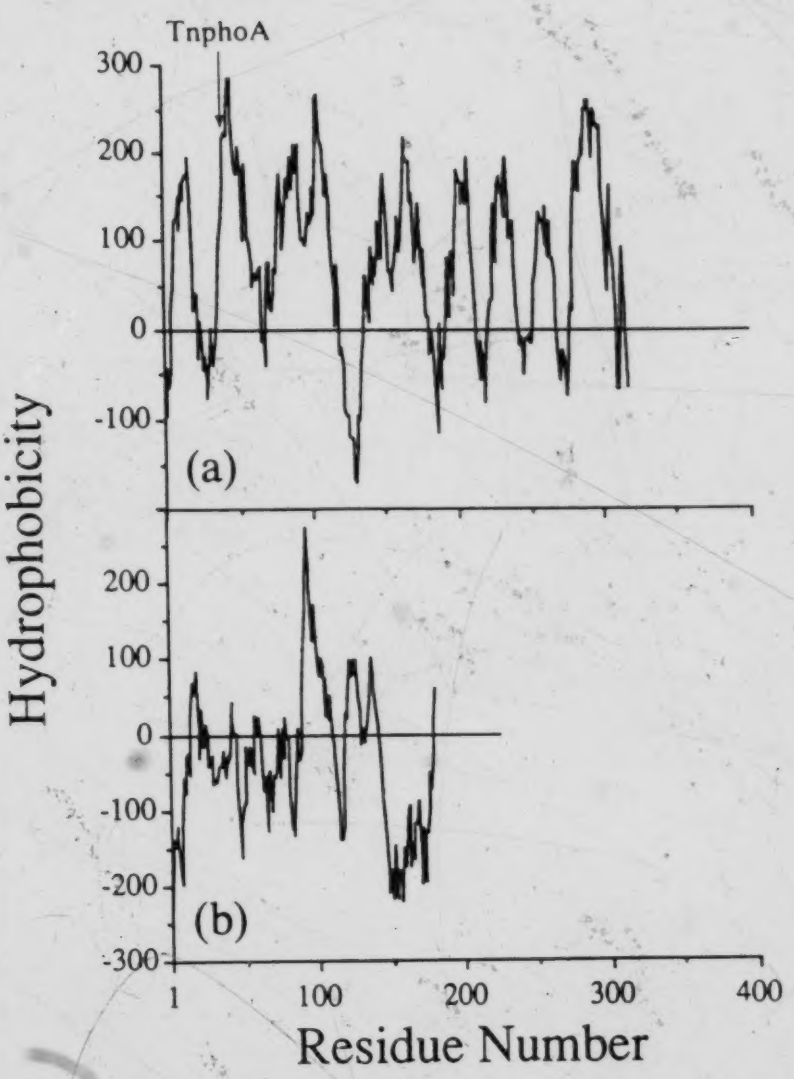


Figure 38. Hydrophobicity plot analysis of the tellurite resistance proteins from pHH1508a.

The deduced amino acid sequences of TehA (a) and TehB (b) (Figure 34) were used to predict the hydrophobicity plots by the algorithms of Kyte and Doolittle (1982). The positive and negative values indicate hydrophobicity and hydrophilicity, respectively. The point of insertion of Tn*phoA* in TehA is shown by an arrow in (a).

to be located in the periplasm since alkaline phosphatase is only active if it is exposed to the periplasmic environment (Manoil, 1990).

Analysis of the amino acid composition of TehA indicates that 55 % of its aa are the relatively hydrophobic IFVLMAT residues (Table 14). It is slightly basic, having 7.8 % basic residues and only 4.2 % acidic residues. Three cysteine residues are found in three of the central hydrophobic domains (Figure 39a). These could be involved in exchange of reducing equivalents within the membrane (Figure 41). There are 7 histidine residues in the sequence, 5 of which are located in the N-terminal half of the protein. The acidic and basic residues of TehA are concentrated in small regions of the polypeptide, separated by long regions which are uncharged (Figure 39). These uncharged sequences may form membrane-spanning domains. Both the N- and C-termini of the polypeptide are highly charged.

The algorithms of Eisenberg et al., (1984b) and Kyte and Doolittle (1982) were used to predict the locations of possible membrane-spanning domains in the TehA protein. Using the method of Eisenberg, 10 possible membrane-spanning regions having mean hydrophobicity values above the threshold of 0.42 were detected (Figure 40). These <H> values ranged from 0.51 to 0.81. These regions were used to construct a model showing the topography of TehA within the inner membrane of the bacterium (Figure 41). In contrast, many of the transmembrane domains predicted by the method of

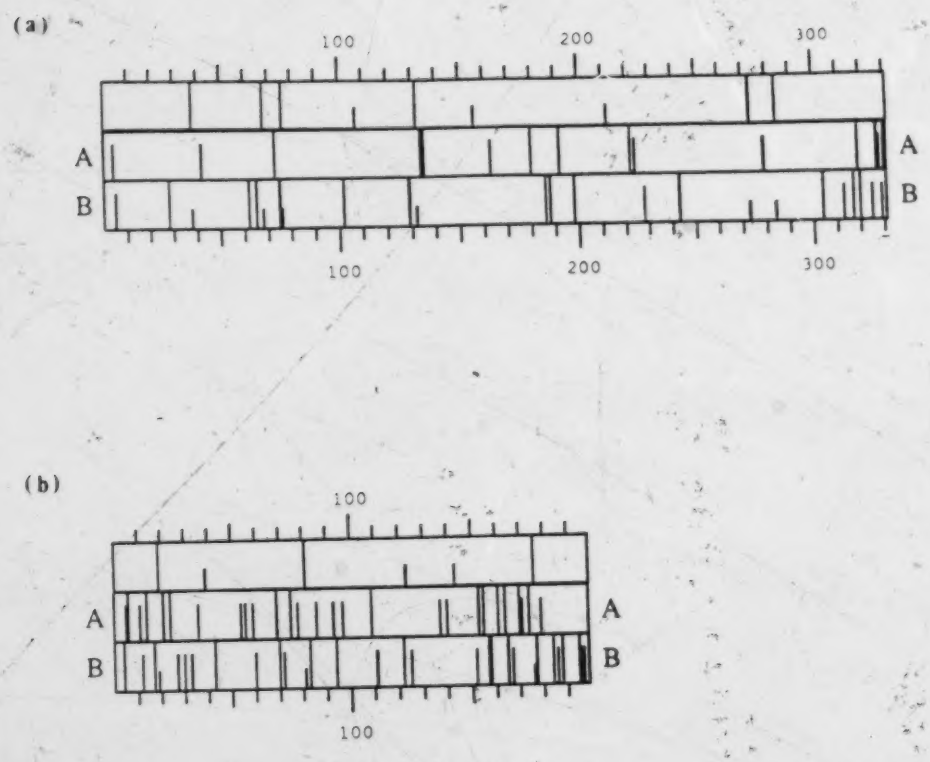


Figure 39. Locations of acidic, basic, Cys and His residues in TehA and TehB.

The positions of cysteine residues (short vertical lines) and histidine residues (full vertical lines) in the sequences of TehA (a) and TehB (b) are indicated in the top rows. The positions of acidic (Glu, full line; Asp, short line) and basic residues (Arg, full; Lys, intermediate; His, short line) in the sequences are indicated in the rows indicated by A and B, respectively.

Table 14. Amino Acid Composition of TehA.

330 Amino Acids MW : 35908 Dalton

			n	n(%)	MW	MW(%)
A	ala	alanine	35	10.6	2486	6.9
C	cys	cysteine	3	0.9	309	0.9
D	asp	aspartic acid	6	1.8	690	1.9
E	glu	glutamic acid	9	2.7	1161	3.2
F	phe	phenylalanine	25	7.6	3676	10.2
G	gly	glycine	31	9.4	1767	4.9
H	his	histidine	6	1.8	822	2.3
I	ile	isoleucine	16	4.8	1809	5.0
K	lys	lysine	5	1.5	640	1.8
L	leu	leucine	53	16.1	5993	16.7
M	met	methionine	10	3.0	1310	3.6
N	asn	asparagine	7	2.1	798	2.2
P	pro	proline	15	4.5	1455	4.1
Q	gln	glutamine	10	3.0	1280	3.6
R	arg	arginine	13	3.9	2029	5.7
S	ser	serine	25	7.6	2175	6.1
T	thr	threonine	18	5.5	1818	5.1
V	val	valine	25	7.6	2476	6.9
W	trp	tryptophan	11	3.3	2046	5.7
X	---	unknown	-	-		
Y	tyr	tyrosine	7	2.1	1141	3.2
Z	---	STOP	-	-		

[a]The number and percentages (by number and by molecular weight) of each amino acid in TehA were determined from the amino acid sequence deduced from the nucleotide sequence (Figure 34) using the MacIntosh computer program DNA Strider (Marck, 1988).

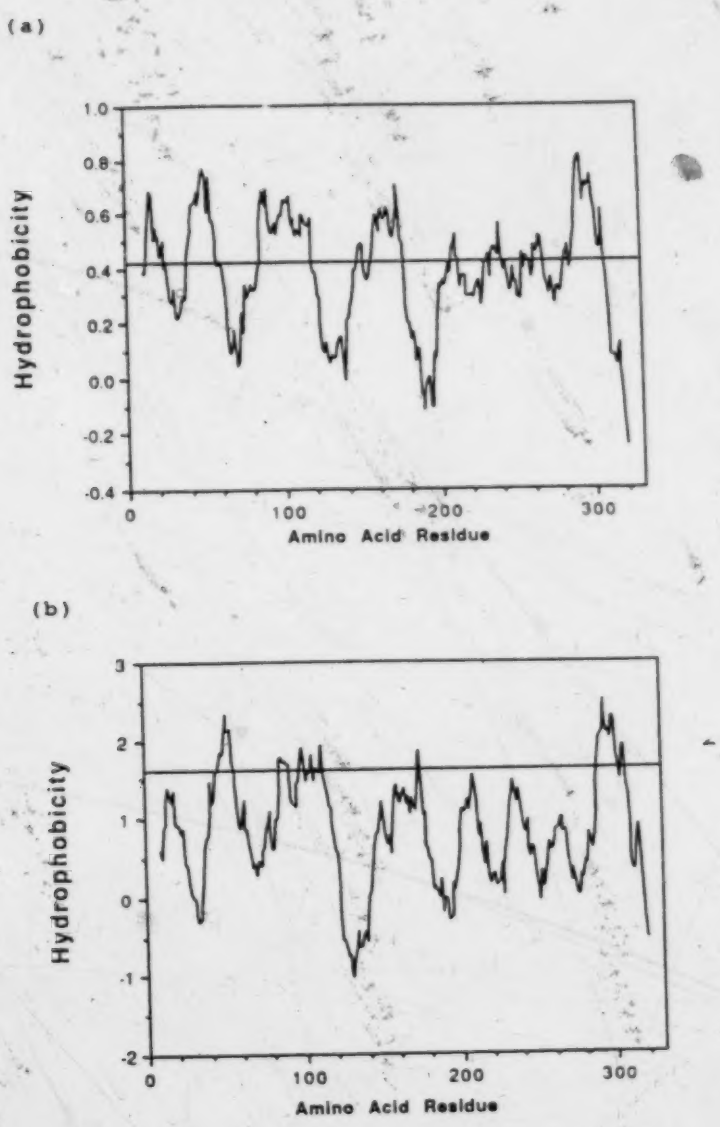


Figure 40. Prediction of membrane-spanning domains in TehA. The transmembrane segment prediction algorithms of (a) Eisenberg et al., (1984b) and (b) Kyte and Doolittle (1982) were used to determine the mean hydrophobicity values in the TehA amino acid sequence over windows of 21 and 19 amino acids, respectively. The threshold values of 0.42 (a) and 1.6 (b) are indicated by dotted horizontal lines.

Eisenberg et al., (1984b) were not predicted by the method of Kyte and Doolittle (1982). Only 5 of these putative membrane-spanning regions had <H> values above the Kyte-Doolittle threshold of 1.6.

As described previously, positively charged residues are more frequently found in the cytoplasmic loops of inner membrane proteins than in the periplasmic loops (von Heijne, 1986). In the proposed model of TehA, the 6 cytoplasmic domains have ratios of positive to negative residues of 1:1, 5:1, 2:2, 3:1, 0:0 and 6:4 (including His). In the periplasmic loops, there are 4 acidic and 4 basic residues. Thus, the distribution of positively charged and negatively charged amino acids in the proposed model fits with previous studies on inner membrane proteins (von Heijne, 1986).

The distribution of Pro residues, however, cannot be used to support the proposed model. Five Pro residues are found in the cytoplasmic regions but none are located in the periplasmic regions (Figure 41). Furthermore, five Pro residues are found in membrane-spanning regions which are periplasmic pointing and only 4 in those which are cytoplasmic pointing. This distribution is the opposite of previous observations on membrane-spanning proteins (von Heijne, 1986).

In contrast to TehA, TehB is relatively hydrophilic, having only 43.1 % hydrophobic aa (IFVLMAT; Table 15). The central region is moderately hydrophobic whereas the N-terminus appears very hydrophilic (Figures 38, 39) It is

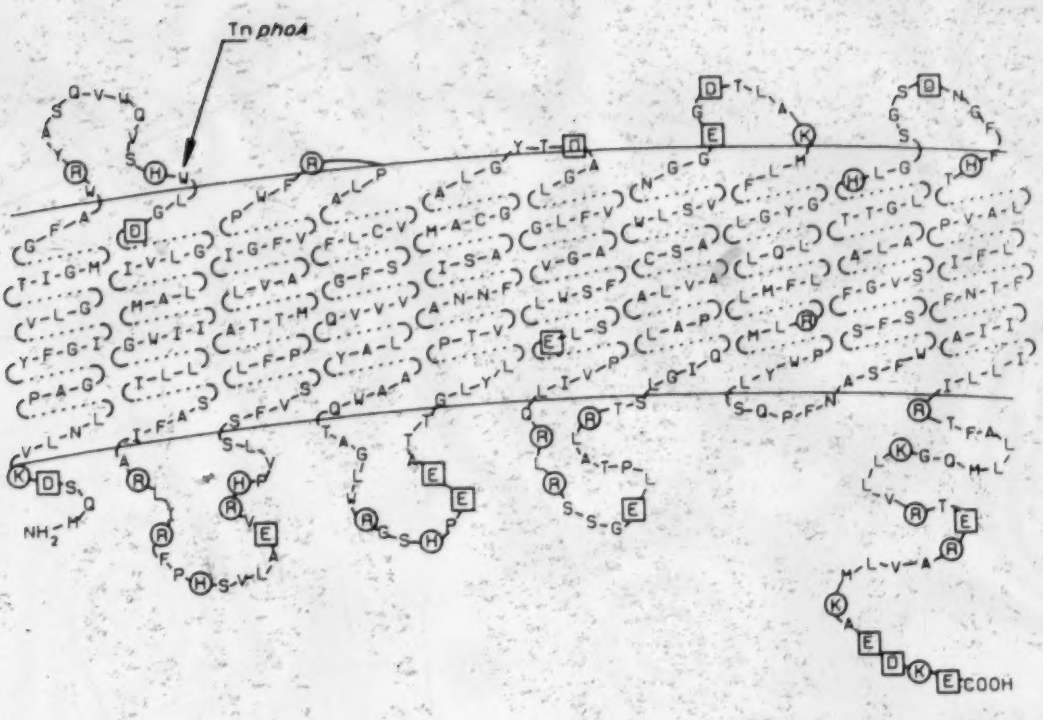


Figure 41. Possible model for the TehA protein.
A possible model for the topology of the Te^R protein TehA in the inner membrane of *E. coli* is shown. Acidic and basic residues in the sequence are indicated by squares and circles, respectively. The point of insertion of Tn*phoA* in a $PhoA^+$ Te^S fusion is indicated.

Table 15. Amino Acid Composition of TehB.

197 Amino Acids MW : 22516 Dalton

			n	n(%)	MW	MW(%)
A	ala	alanine	15	7.6	1065	4.7
C	cys	cysteine	3	1.5	309	1.4
D	asp	aspartic acid	14	7.1	1610	7.2
E	glu	glutamic acid	13	6.6	1677	7.5
F	phe	phenylalanine	8	4.1	1176	5.2
G	gly	glycine	13	6.6	741	3.3
H	his	histidine	3	1.5	411	1.8
I	ile	isoleucine	10	5.1	1130	5.0
K	lys	lysine	13	6.6	1665	7.4
L	leu	leucine	19	9.6	2148	9.5
M	met	methionine	6	3.0	786	3.5
N	asn	asparagine	15	7.6	1710	7.6
P	pro	proline	5	2.5	485	2.2
Q	gln	glutamine	2	1.0	256	1.1
R	arg	arginine	14	7.1	2185	9.7
S	ser	serine	5	2.5	435	1.9
T	thr	threonine	13	6.6	1313	5.8
V	val	valine	14	7.1	1386	6.2
W	trp	tryptophan	2	1.0	372	1.7
X	---	unknown	-	-		
Y	tyr	tyrosine	10	5.1	1630	7.2
Z	---	STOP	-	-		

[a]The number and percentages (by number and by molecular weight) of each amino acid in TehB were determined from the amino acid sequence deduced from the nucleotide sequence (Figure 34) using the MacIntosh computer program DNA Strider (Marck, 1988).

also slightly basic, having 13.7 % acidic and 20.3 % basic aa which are spread throughout the sequence (Figure 39). It has 3 Cys and 3 His residues which are spaced throughout the polypeptide (Figure 39).

3.20 Comparison of the IncHII Te^R Sequence with other sequences.

The DNA sequences and derived amino acid sequences of the Te^R genes of pHH1508a were compared with those of the IncPα plasmid, RK2; however, no significant homology could be detected. This confirms the DNA-DNA hybridization studies with the IncPα Te^R determinant and suggests that the two Te^R determinants are unrelated.

No homology was found between the sequences of the Te^R determinant from the IncHII plasmid, pHH1508a, and the Te^R determinant from the IncHI2 plasmid, pMER610. Although TehA and ORF3 are very close in size and in the number of proposed membrane-spanning domains, they have little, if any, similarity in their sequences. As described earlier, ORF3 is more highly charged and is slightly acidic (11 % acidic, 8.7 % basic residues) compared to TehA (4.2 % acidic, 7.8 % basic). This suggests that the two proteins are not functionally related.

The sequence was used to search recent DNA and protein data banks. Again, no significant homology could be found. Some sequence similarity was found between the aa sequences

of TehA and the outer membrane protein, hemolysin B (HlyB) from *Escherichia coli* (Wagner et al., 1983; Felmlee et al., 1985). Over a 62 aa hydrophobic region of TehA, there was 40.6 % homology with a highly hydrophobic region of HlyB (Figure 42).

A consensus sequence (**G....GKT/S**) has been identified in ATP-binding proteins (Higgins et al., 1986). A region with some similarity to this consensus sequence is found in TehB starting at aa residue 27 (K....**GKT**) (Figure 34).

In contrast to the IncPα TelB protein, no closely spaced cysteine residues are found in either TehA or TehB. However, as noted earlier, TehA has three cysteines in three of the proposed membrane-spanning domains in the central portion of the protein (Figure 41). TehB also has three Cys residues (Figure 39) which could be involved in the formation of disulfide bridges or the binding of metals or reducing equivalents.

3.21 Overproduction of Te^R proteins using the T7 system.

The plasmids pT7-5 and pT7-6 (Ap^R) have a promoter for the RNA polymerase from bacteriophage T7 (ϕ10) followed by multiple cloning sites which are in opposite orientations (Figure 43; Tabor and Richardson, unpublished maps). The plasmid pGP1-2 (Km^R) carries the gene for T7 RNA polymerase under the control of the inducible λ P_L promoter and the gene for the temperature-sensitive repressor, c*I857*. Genes placed

```
TehA    11    AGYFGIVLGTIGMGFAWRYASQVWQVSHWLGDGLVILAMI     50
              **|   ** ***          *  |  ***  ***   |
HlyB   366    AGFKVTVLATIGQQGIQLIQKTVMIINLWLGAHLVISGDL   405

TehA    51    IWGLLTSAF..IARLIRFPHSVLA     72
                * *  **  |*  *  *  **
HlyB   406    SIGQLI.AFNMLAGQIVAPVIRLA    428
```

Figure 42. Similarity between TehA and HlyB.

A region of sequence similarity between TehA and Hemolysin B from *Escherichia coli* (Felmlee et al., 1985) is shown. Identity between aas in the two proteins is indicated by '*'. Conservative aa changes are indicated by '|' (Appendix 3). Spaces inserted in the sequence to optimize the alignment are indicated by '.'. The start and end positions in the aa sequences of the proteins are indicated at the left and right of each line. Percent similarity (40.6) was determined by adding up the number of identical and conserved aa residues and dividing by the total length (including spaces) of the compared region in TehA.

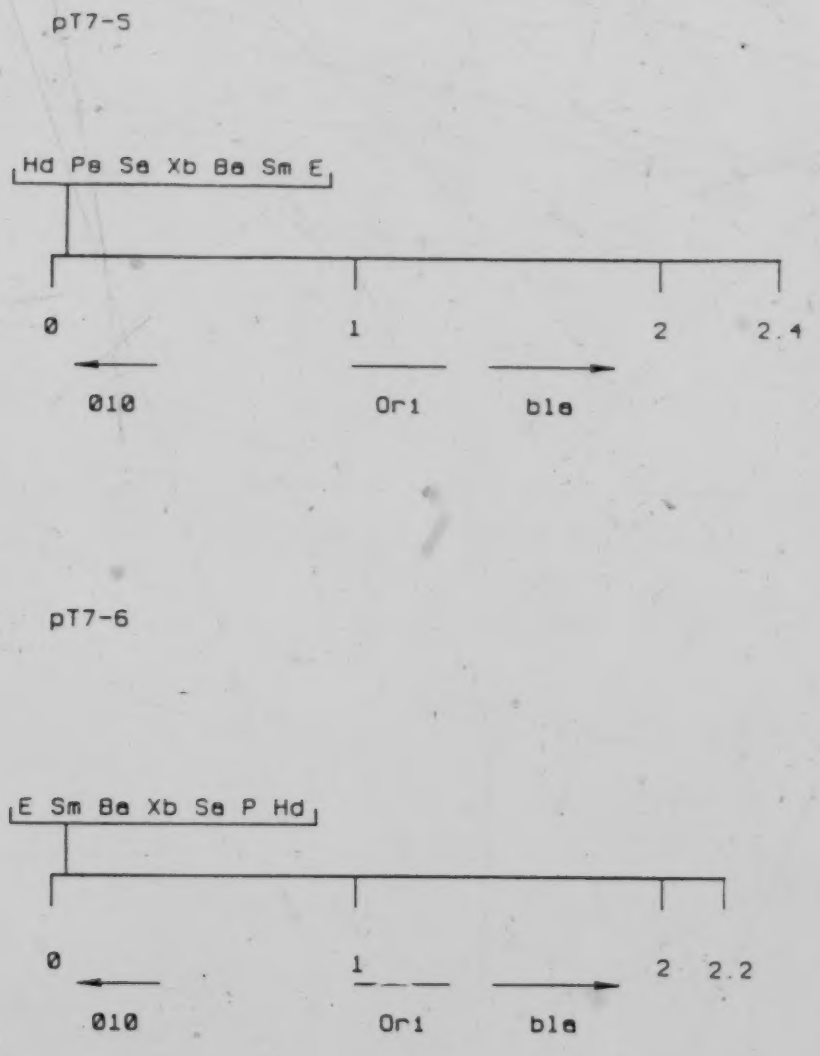


Figure 43. Restriction maps of pT7-5 and pT7-6.
The relative positions of the restriction endonuclease sites in the multiple cloning regions of the plasmids pT7-5 and pT7-6 are shown. pT7-5 and pT7-6 are identical except for the orientation of the multiple cloning sites with respect to the T7 promoter. The directions of transcription from the T7 promoter and of the β-lactamase gene are indicated by arrows (S. Tabor and C. Richardson, unpublished data).

under the control of the T7 promoter are induced by raising the temperature to 42°C which allows the expression of T7 RNA polymerase from the plasmid pGP1-2 carried in the same cell (Tabor and Richardson, 1985).

The IncPα Te^R region of pDT1558 was placed under the control of the T7 promoter by ligating the *Bam*HI/*Hin*dIII fragment of pDT1555 into the corresponding sites of pT7-5, creating the plasmid pDT1968 (Figure 44a). The plasmid pDT2031 carrying the *kilA* and *telA* genes of RK2Te^R was constructed by ligating the *Xho*I/*Bam*HI fragment of pDT1555 into *Sal*I/*Bam*HI digested pT7-5 (Figure 44b). Finally, the plasmid pDT2034 carrying the *kilA* gene and part of the *telA* gene was constructed by ligating the *Bam*HI/*Sma*I fragment of pDT1555 into similarly digested pT7-6 (Figure 44c).

The IncHII Te^R region of pDT1364 was subcloned to allow for controlled expression in the T7 system. The plasmid pDT1869 was constructed by ligating the *Sal*I/*Pst*I fragment of pDT1557 into similarly digested pUC8 (Figure 45a). The Te^R plasmid pDT1947 was constructed by ligating the *Eco*RI/*Xho*I fragment of pDT1869 into *Eco*RI/*Sal*I digested pUC8 (Figure 45b). Finally, the Te^R plasmid pDT1971 was constructed by ligating the *Eco*RI/*Xho*I fragment of pDT1869 into *Eco*RI/*Sal*I digested pT7-6 (Figure 45c).

Proteins determined by the Te^R regions of RK2 and pHH1508a were overproduced by induction at 42°C. Whole cell lysates were subjected to SDS-PAGE and compared with whole cell lysates from *E. coli* (pT7-5). On Coomassie-blue stained

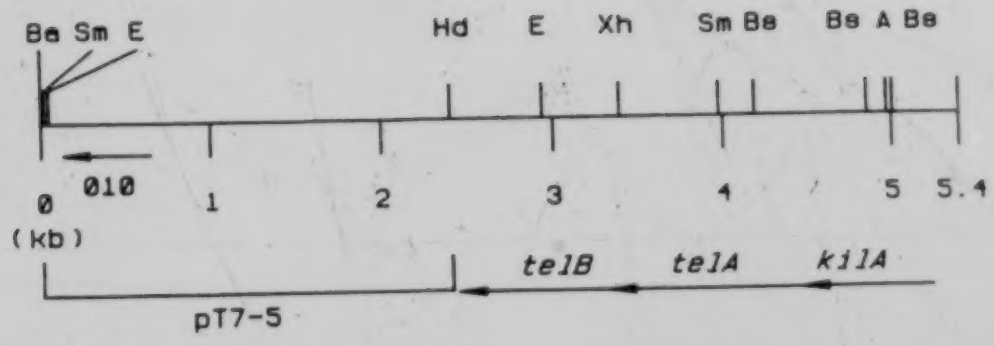
Figure 44. Restriction map of pT7 IncPα Te^R subclones.

(a) The Te^R plasmid pDT1968 was constructed by ligating the *Bam*HI/*Hin*dIII fragment of pDT1555 carrying the Te^R region of RK2Te^R into the corresponding sites of pT7-5.

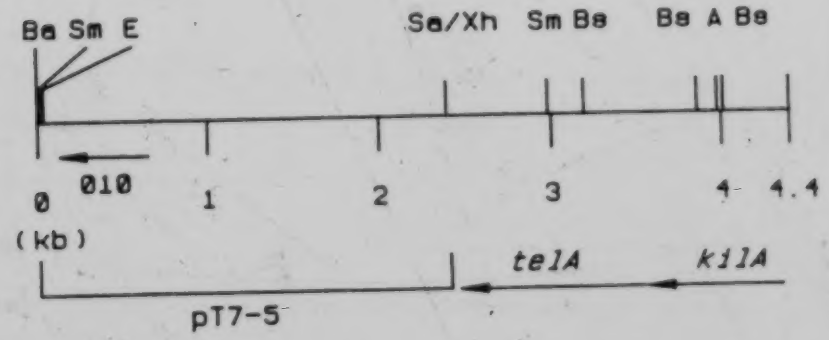
(b) The plasmid pDT2031 carrying the *kilA* and *telA* genes of RK2Te^R was constructed by ligating the *Xho*I/*Bam*HI fragment of pDT1555 into *Sal*I/*Bam*HI digested pT7-5.

(c) The plasmid pDT2034 carrying the *kilA* gene and part of the *telA* gene was constructed by ligating the *Bam*HI/*Sma*I fragment of pDT1555 into similarly digested pT7-6.

(a) pDT1968



(b) pDT2031



(c) pDT2034

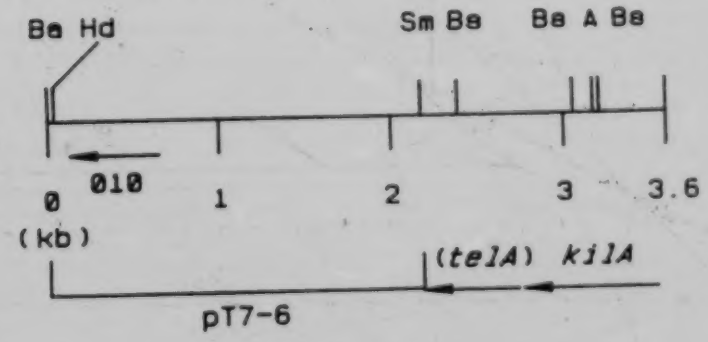
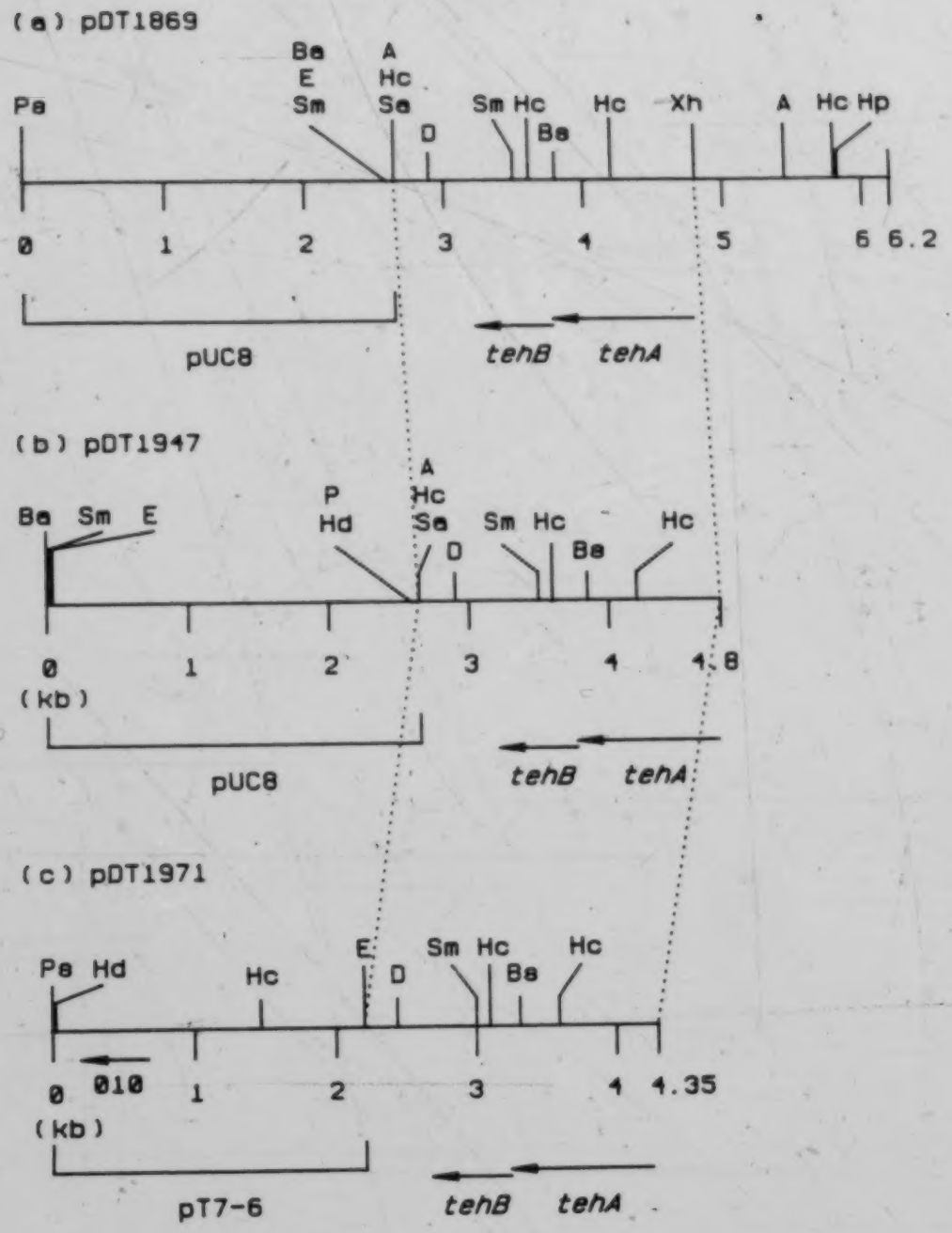


Figure 45. Restriction map of IncHII Te^R subclones.

(a) The Te^R plasmid pDT1869 was constructed by ligating the *SalI/PstI* fragment of pDT1557 carrying the Te^R region of pHH1508a to similarly digested pUC8.

(b) The Te^R plasmid pDT1947 was constructed by ligating the *EcoRI/XhoI* fragment of pDT1869 carrying the Te^R region of pHH1508a to *EcoRI/SalI* digested pUC8 DNA.

(c) The Te^R plasmid pDT1971 was constructed by ligating the *EcoRI/XhoI* fragment of pDT1869 carrying the Te^R region of pHH1508a to *EcoRI/SalI* digested pT7-6.


(a) pDT1869
Ba
E
Sm
A
Hc
Se
Ps
D
Sm Hc
Ba
Hc
Xh
A
Hc Hp
0
1
2
3
4
5
6 6.2
pUC8
tehB
tehA
(b) pDT1947
Ba Sm E
P
Hd
A
Hc
Se
D
Sm Hc
Ba
Hc
0
1
2
3
4
4.8
(kb)
pUC8
tehB
tehA
(c) pDT1971
Ps Hd
Hc
E
D
Sm Hc
Ba
Hc
0
010
1
2
3
4
4.35
(kb)
pT7-6
tehB
tehA

gels, only one overproduced protein could be detected from each Te^R determinant (Figure 46). A protein with an apparent molecular mass of 42 kDa was expressed by pDT1968 which is probably the TelA protein. The plasmid pDT1971 expressed a unique polypeptide of 23 kDa which is probably TehB.

The membrane proteins, TelB and TehA, could not be detected. Therefore, the more sensitive method involving labelling proteins with L-[^{35}S]methionine, was used. In addition, rifampicin was added. This antibiotic inhibits the synthesis of mRNA by *E. coli* RNA polymerase but not T7 RNA polymerase (Chamberlin et al., 1970).

After labelling overproducing cells with L-[^{35}S]methionine, the whole cell lysates were subjected to SDS-PAGE. The autoradiogram showed that three proteins were expressed by cells carrying pDT1968 which contains the entire *kilA-telAB* region (Figure 47). These proteins had molecular masses of 48, 31 and 30 kDa. Cells carrying pDT2031, which encodes only the *kilA* and *telA* genes, did not express the 30 kDa protein, indicating that this is the TelB protein. Cells carrying pDT2034, containing all of the *kilA* gene and part of the *telA* gene did not express the 48 kDa protein indicating that this is TelA. The 31 kDa protein was still produced and appears to be KilA, and a new band of 28 kDa was seen which appears to be a truncated form of the TelA protein. The sizes of the proteins overproduced using this system are very close to those predicted from the nucleotide sequence.

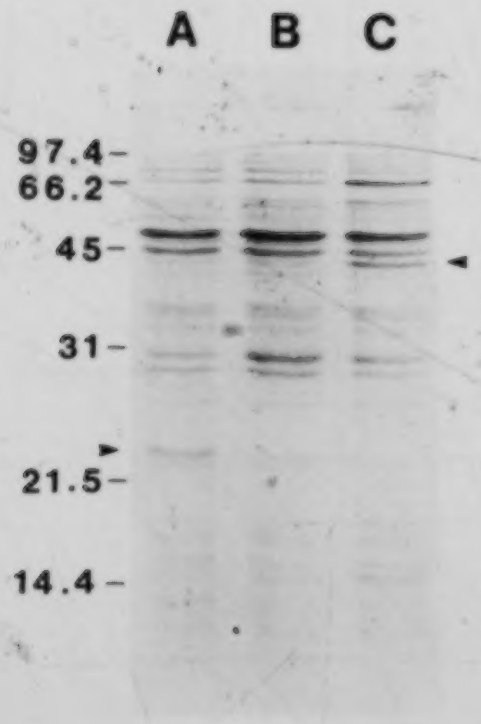
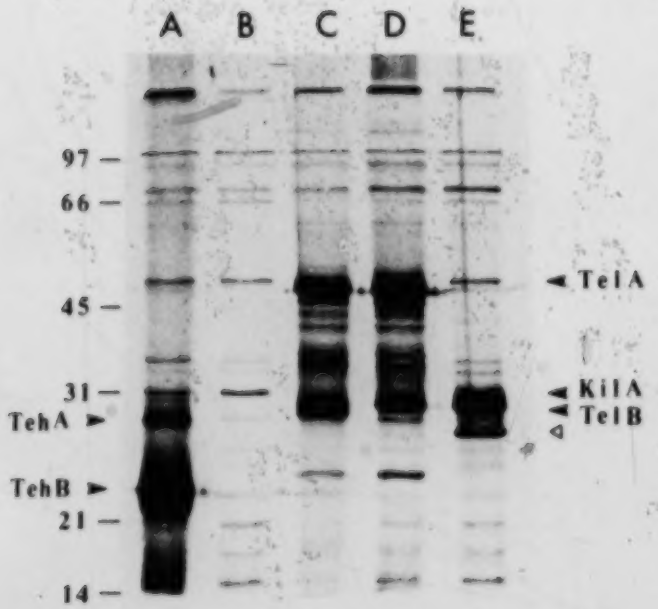


Figure 46. Detection of Te^R proteins on a Coomassie Blue-stained SDS-PAGE gel.

Coomassie Blue-stained SDS-PAGE gel showing the proteins expressed in cells containing each recombinant plasmid after induction of expression from the T7 promoter as described in Materials and Methods (section 2.23). Lanes contain cell lysates of *E. coli* K38 (pGP1-2) carrying the following plasmids: (A) pDT1971; (B) pT7-5; (C) pDT1968. The IncHII Te^R protein TehB is indicated on the left by an arrow and has an estimated size of 23 kDa. The IncPα Te^R protein TelA is indicated on the right by an arrow and has an estimated size of 42 kDa. The molecular masses and positions of the marker proteins are indicated on the left in kilodaltons.

Figure 47. Autoradiogram of overproduced Te^R proteins.

Autoradiogram showing the proteins expressed in cells carrying T7 plasmids after induction as described in Materials and Methods (section 2.23). Lanes contain cell lysates of *E. coli* K38 (pGP1-2) carrying the following plasmids: (A) pDT1971 (carrying the entire IncHII Te^R region); (B) pT7-5; (C) pDT1968 (carrying the entire IncPα Te^R region); (C) pDT2031 (carrying *kilA* and *telA*); (D) pDT2034 (carrying *kilA* and part of *telA*). The IncPα Te^R proteins TelA, KilA, and TelB, are indicated on the right by arrows and have estimated sizes of 48, 31 and 30 kDa, respectively. A truncated TelA protein is indicated on the right by an open triangle. The IncHII Te^R proteins, TehA and TehB, are indicated to the left by arrows. These proteins have estimated molecular masses of 28 and 23 kDa, respectively, based on their migratory distances. The molecular masses and positions of the marker proteins are indicated on the left in kilodaltons.


A
B
C
D
E
97
66
45
31
21
14
TehA
TehB
TelA
KilA
TelB

Expression of the IncHII Te^R genes carried on pDT1971 was induced as described above. After labelling with L-[^{35}S]methionine, two overproduced proteins were detected in cells carrying the Te^R determinant of pHHI508a which were not seen in lysates of cells carrying pT7-5 (Figure 47). These proteins had molecular masses of 28 and 23 kDa based on their migratory distances in SDS-PAGE. The 23 kDa size corresponds exactly to the size of TehB predicted from the sequence and seen in *in vitro* transcription-translation. The second polypeptide, presumably TehA, has a much smaller apparent size (28 kDa) than predicted from the deduced aa sequence (36 kDa). This may be due to the large number of hydrophobic aa in TehA.

The autoradiogram suggests that there is much more TehB than TehA present in overproducing cells. Both polypeptides have the same proportion of methionine residues in their sequences (Table 14) and therefore should be labelled with L-[^{35}S]methionine to the same extent. The difference may be due to some of the highly hydrophobic TehA not entering the stacking or separating gels since dark bands were seen at these two positions on the gel (Figure 47). Alternatively, this could be due to inefficient translation of *tehA* which has a poor ribosome-binding sequence (Figure 34) or due to specific degradation of the *tehA* mRNA.

3.22 Electron microscopy of Te^R bacteria.

Bacteria carrying the Te^R determinant of pDT1364 which had been grown on media containing 50 µg/ml tellurite were examined by electron microscopy. Electron dense intracellular granules were seen in both unfixed unstained whole cells and thin sections of bacteria fixed in glutaraldehyde and stained with uranyl acetate (Figures 48, 49). These black particles appeared to be associated with the bacterial membranes (Figure 49). Electron spectroscopic imaging has shown that these black granules contain only metallic tellurium and not compounds of tellurium containing either potassium or oxygen (Taylor et al., 1988).

In cells in which the outer membrane had separated from the inner membrane, tellurium crystals were found inside the inner membrane (Figure 50). Occasionally crystals of tellurium were seen in the surrounding medium, however, this was usually near cells which appeared to have lysed. The amount of electron dense material in cells carrying either pHH1508a or pDT1364 was approximately 10 times that in cells carrying either RP4 or pDT1366 (Taylor et al., 1988).

Te^S *E. coli* which were grown in LB and then exposed to a high concentration of tellurite (100 µg/ml) also formed these black tellurium granules within the cytoplasm (Figure 51). The shape and position of these crystals within the Te^S bacterium was similar to that in the Te^R bacteria grown in the presence of tellurite.

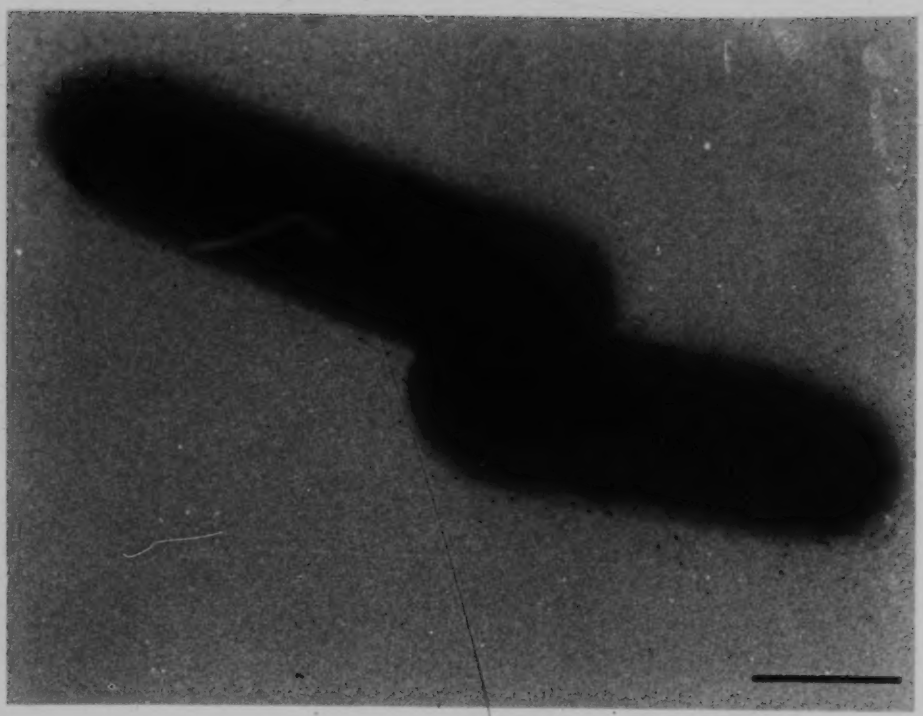


Figure 48. Electron micrograph of whole unfixed bacteria grown on tellurite.

E. coli cells carrying the plasmid pDT1364 were grown on media containing potassium tellurite (50 µg/ml). Whole unfixed, unstained bacteria were examined by electron microscopy as described previously (Taylor et al., 1988). The black bar represents 1 µm.

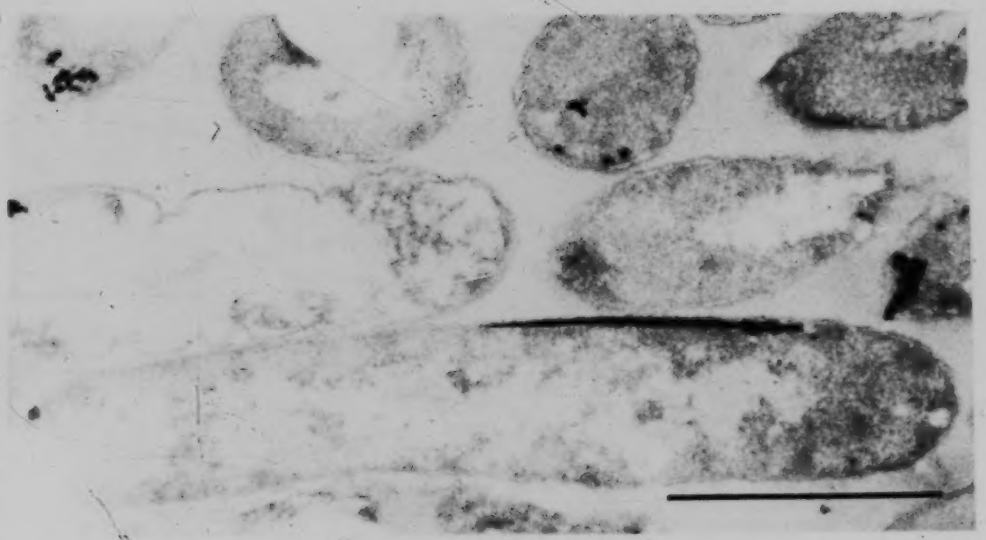


Figure 49. Electron micrograph of thin-sections of Te^R bacteria.

E. coli cells carrying the plasmid pDT1364 were grown on media containing potassium tellurite (50 µg/ml). The bacteria were fixed in glutaraldehyde and stained with uranyl acetate. Thin-sections were examined by electron microscopy as described previously (Taylor et al., 1988). The black bar represents 1 µm.

Figure 50. Location of tellurium within bacterial cells. *E. coli* (RP4Te^R) cells which were grown on tellurite media were fixed in glutaraldehyde and stained with uranyl acetate. Thin sections were examined by electron microscopy as described previously (Taylor et al., 1988). The black bar represents 0.25 µm.

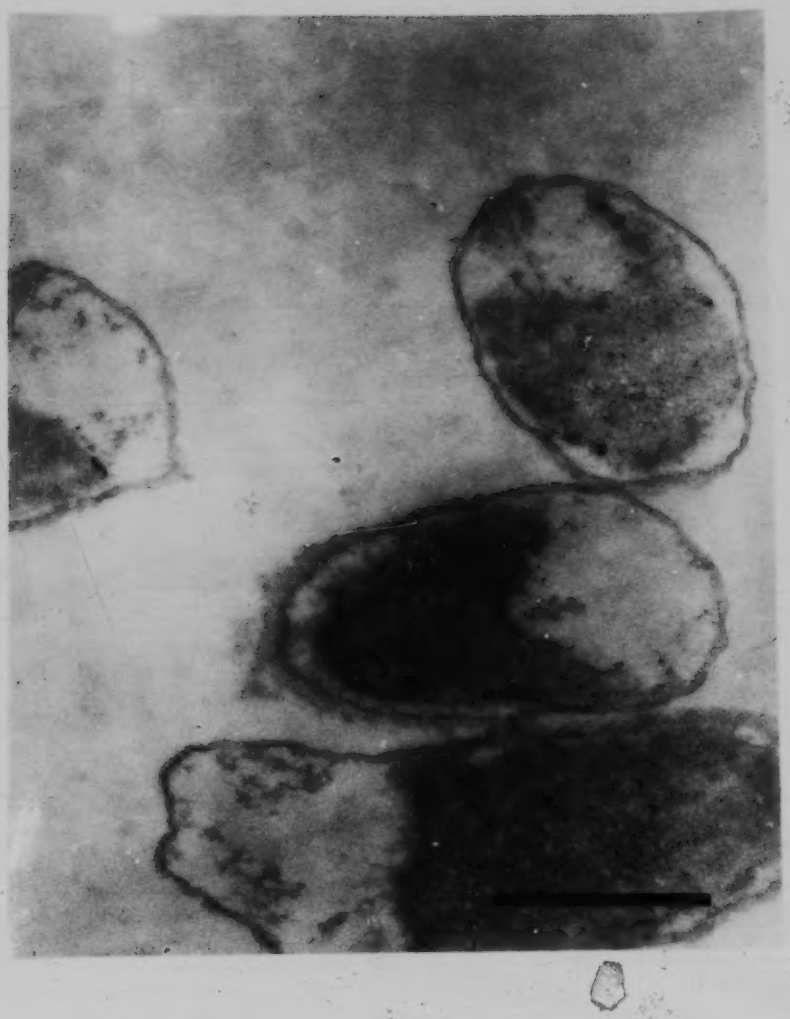


Figure 51. Electron micrograph showing tellurium deposited within Te^S *E. coli*.

Tellurite-sensitive *E. coli* JM83 (pUC8) cells were grown in LB and then incubated with tellurite (100 µg/ml) until a black color formed. The bacteria were fixed in glutaraldehyde, stained with uranyl acetate and examined by electron microscopy. The black bar represents 0.5 µm.

3.23 Media-conditioning by Te^R bacteria.

The ability of *E. coli* carrying the Te^R genes from pHH1508a and RK2Te^R to detoxify tellurite-containing media was investigated. In agar-conditioning experiments, very little difference in the ability of *E. coli* JM83 carrying pUC8, pDT1558 or pDT1364 to detoxify the media could be seen. The Te^S strain, *E. coli* JM83, was not able to grow significantly closer to the tellurite disc near regions of growth of *E. coli* JM83 (pDT1558) or *E. coli* JM83 (pDT1364) compared to *E. coli* JM83 (pUC8). Therefore, tellurite broth conditioning experiments were performed.

Bacteria containing the plasmid pDT1558, RP4, or pDT1364 were grown in BHI broth containing tellurite and the amount of tellurite left in the broth was estimated by measuring the toxicity of the sterile filtrate towards Te^S *E. coli* JM83. Bacteria containing either RP4 or pDT1558 only reduced the toxicity of the media by one half in this method (Table 16). The lack of growth of *E. coli* JM83 in the filtrate of these cultures was not due to depletion of nutrients or production of toxic by-products of metabolism since it was able to grow in the filtrate of *E. coli* JM83 (pUC8) to which tellurite had not been added. These results suggest that the Te^R determinant from RP4 may result in decreased uptake or increased efflux of tellurite from the bacterial cells.

In contrast, bacteria carrying pDT1364 repeatedly reduced the toxicity of the tellurite broth by at least 128-fold

Table 16. Media-conditioning by Te^R bacteria.

Filtrate of culture:	Dilutions of filtrate:												
	1 (no JM83 added)[a]	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/1024 (no JM83 added)
pUC8 (no Te)	-	+	+	+	+	+	+	+	+	+	+	+	-
BHIB + Te_{64}[b]	-	-	-	-	-	-	-	-	+	+	+	+	-
pUC8 + Te_{64}[c]	-	-	-	-	-	-	-	-	+	+	+	+	-
pDT1558 + Te_{64}	-	-	-	-	-	-	-	+	+	+	+	+	-
RP4 + Te_{64}	-	-	-	-	-	-	-	+	+	+	+	+	-
pDT1364 + Te_{64}	-	+	+	+	+	+	+	+	+	+	+	+	-

+ and - indicate the presence or absence of growth of *E. coli* JM83 which was added to the indicated dilution of the culture filtrate.

[a]Control to check sterility of filtrate.

[b]Te_{64} indicates potassium tellurite was added to the broth to a final concentration of 64 µg/ml before overnight incubation with the indicated strain.

[c]*E. coli* JM83 (pUC8) did not grow in the presence of Te_{64}

(Table 16). No evidence of contamination in any of the filtrates was seen. This result suggests that the Te^R determinant on this plasmid mediates a detoxification mechanism.

3.24 Measurement of tellurite reductase activity.

During studies on a tellurite reductase from *Thermus thermophilus*, Chiong et al., (1988) found that reduction of tellurite to black tellurium metal could be observed *in situ* in nondenaturing isoelectric focusing gels. Therefore, protein extracts were prepared from bacteria carrying the Te^R determinant of either RP4 or pHH1508a. These were loaded onto a nondenaturing PAGE gel. After electrophoresis, the gel was incubated in the presence of 1 mM potassium tellurite and 1 mM NADH, a potential electron donor, as described by Chiong et al., (1988). No evidence of tellurite reduction in the gel was seen, suggesting that any tellurite reductases present in these cells are not active under these conditions.

The tellurite resistance expressed by pDT1364 and pDT1558 was tested for any possible requirement for thioredoxin, a potential electron donor, by transforming the plasmids into *E. coli* DL16 which has a mutation in the thioredoxin gene (Mark et al., 1977). Both plasmids expressed resistance to tellurite in this strain indicating that thioredoxin is not necessary for this function.

By visual examination, the rate of reduction of tellurite to black metallic tellurium was faster in *E. coli* carrying the plasmid pDT1364 compared to *E. coli* carrying either pUC8 or pDT1558. However, upon French Press lysis of the bacterial cells, the rate of this reaction was significantly decreased. Therefore, an appropriate method of measuring quantitatively the rate of formation of black metallic tellurium by whole cells was required. Because of the problem of light scattering by whole cells, which was further compounded by the presence of black tellurium crystals, various filters in a Klett colorimeter were tested for their usefulness in measuring the rate of formation of black reduced tellurium by whole cells (Table 17). Two cultures, *E. coli* (pDT1364) grown in the presence of tellurite and *E coli* (pUC8) grown in the absence of tellurite, were tested with each of 6 filters. Filters 42 and 50 both gave large differences in readings between the two cultures. Filter 50 was used for further testing since its reading for the culture grown in the absence of tellurite was much lower than that obtained with Filter 42. The readings with this filter were close to being proportional when dilutions of the cultures were prepared (Table 18); therefore, this filter was used to estimate the rate of reduction of tellurite in further studies.

The rates of reduction of tellurite by *E. coli* JM83 carrying pUC8, pDT1364 or pDT1558 were compared (Figure 52). Cells carrying the IncHII Te^R plasmid, pDT1364, reduced

Table 17. Measurement of tellurite reduction with different Klett filters.

Filter #	Klett Units		Difference in Klett units
	E. coli (pDT1364) (+Te)	*E. coli* (pUC8) (no Te)	
42	151.5	79	72.5
50	125	55	70
54	121	53	68
59	111	45	66
62	99	40	59
66	90	34.3	55.7

Table 18. Measurement of tellurite reduction with Klett Filter 50.

Dilution	Klett Units		Difference in Klett Units
	E. coli (pDT1364) (+Te)	*E. coli* (pUC8) (no Te)	
1/5	242	102	140
1/10	127	55.3	71.7
1/20	61.2	27.3	33.9

tellurite much faster than cells carrying pUC8 or the IncPα Te^R plasmid, pDT1558. This began immediately upon addition of tellurite which is consistent with constitutive production of the Te^R proteins.

In contrast, cells carrying the plasmids pT7-5, pDT1971 (HII Te^R determinant in pT7-6), or pDT1968 (IncPα Te^R in pT7-5) which had been induced as described above, reduced tellurite more slowly than cells carrying the pUC8 recombinant plasmids (Figure 53). This suggests that the large amount of Te^R proteins being produced were not active in tellurite reduction.

E. coli (pDT1364) bacterial cells grown in LB were centrifuged and resuspended in a minimal medium containing various carbon substrates and tellurite was added. In comparison to the rates of reduction of tellurite to tellurium observed when the cells were incubated in LB or glycerol buffer, formation of the black tellurium metal was significantly slower when *E. coli* (pDT1364) was resuspended in a glucose buffer and moderately slower when no carbon substrate was present.

Reduction of tellurite by *E. coli* JM83 carrying pUC8, pDT1364 or pDT1558 was eliminated when potassium cyanide, which inhibits electron transport, was added to the culture at the same time that potassium tellurite was added (Figure 54). In contrast, dinitrophenol, which dissipates the proton gradient but does not inhibit electron transport, had less effect on the reduction of tellurite by the cells. These

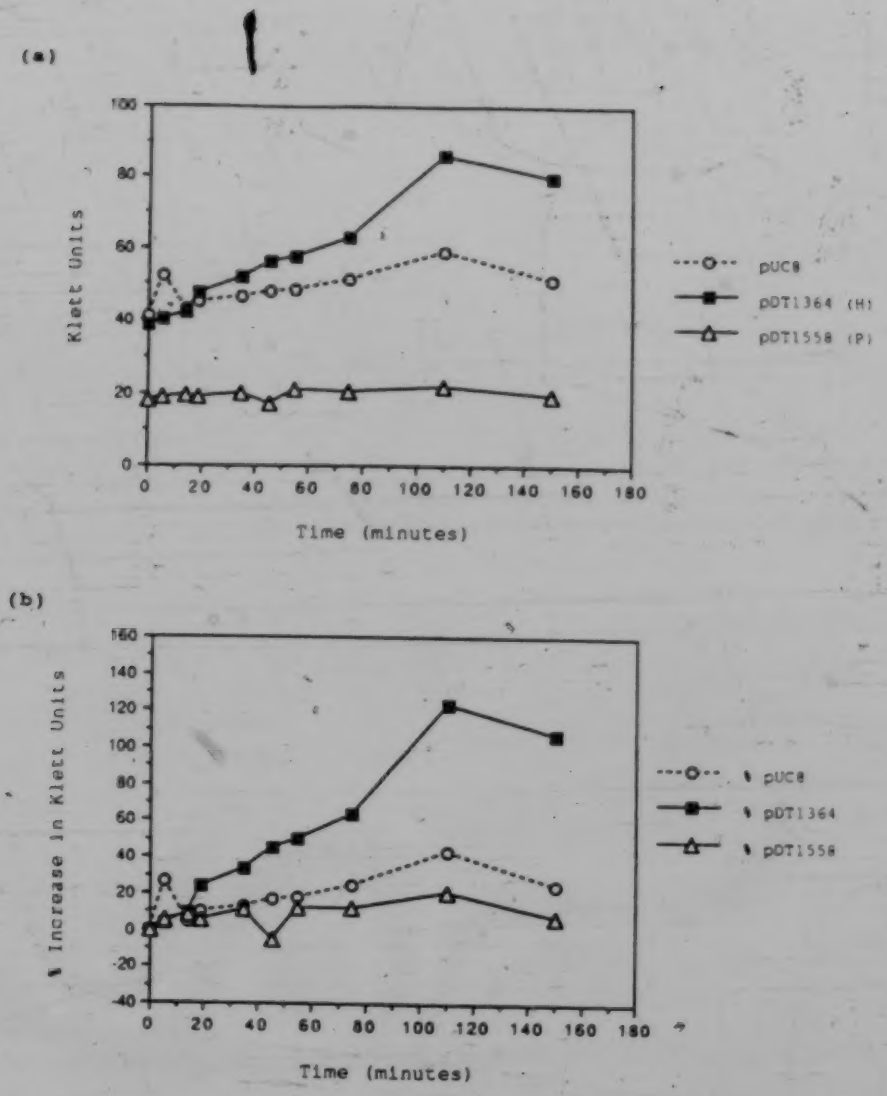


Figure 52. Reduction of tellurite by Te^R and Te^S *E. coli*. The extent of reduction of tellurite to black metallic tellurium was estimated using a Klett colorimeter. *E. coli* cells carried pUC8 (Te^S control) or either of the Te^R clones pDT1364 or pDT1558, as indicated. (a) Klett units of 1/10 dilution of culture after indicated time of incubation with 100 µg/ml tellurite. (b) Results from (a) shown as the percentage increase compared to Klett readings for each culture at the start of the experiment.

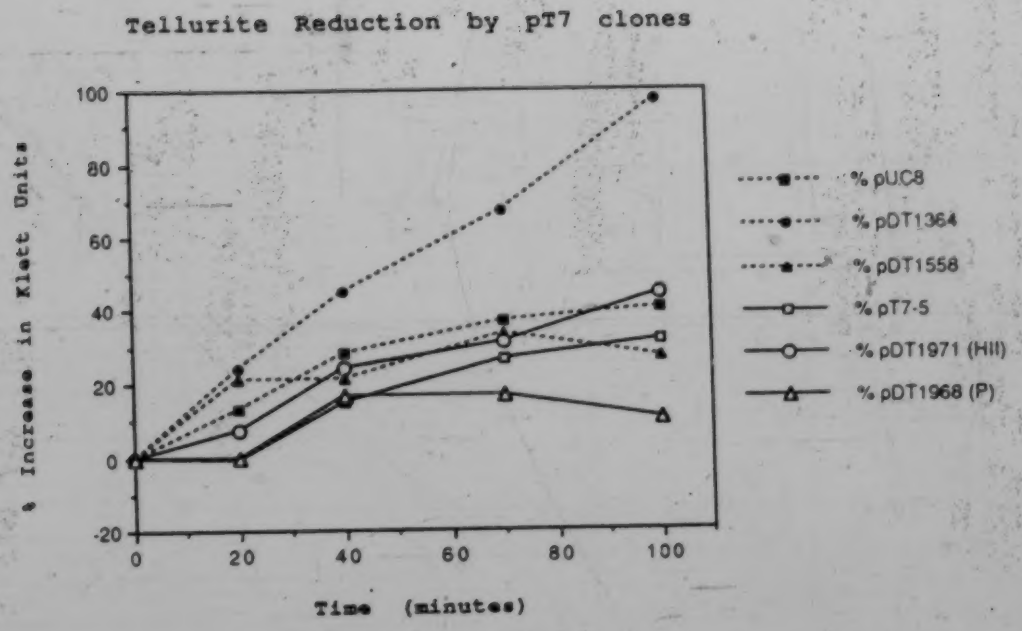


Figure 53. Reduction of tellurite by T7 overexpression cells. Bacteria were induced to overexpress the Te^R proteins as described in materials and methods (Section 2.23). The rate of reduction of tellurite to black metallic tellurium was estimated using a Klett colorimeter. *E. coli* cells carry either pT7-5 (Te^S control) or either of the Te^R clones, pDT1968 or pDT1971. Results are shown as % increase in Klett readings compared to initial reading at time of addition of tellurite (100 µg/ml).

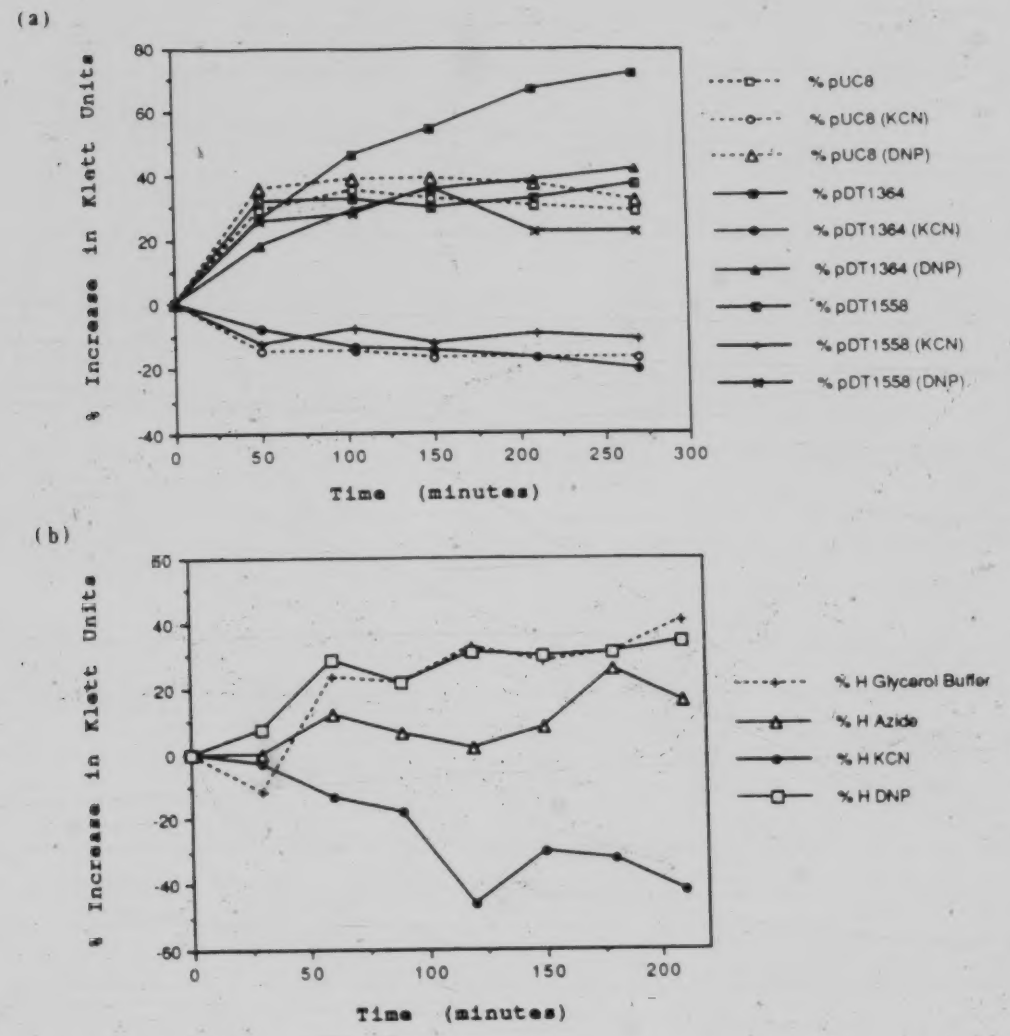


Figure 54. Reduction of tellurite by Te^R and Te^S bacteria in the presence of metabolic inhibitors.

The effect of the metabolic inhibitors sodium azide, potassium cyanide, and dinitrophenol on the rate of tellurite reduction was measured using a Klett colorimeter. (a) Effect of KCN and DNP on the % increase in Klett readings for *E. coli* cells carrying plasmid pUC8, pDT1558, or pDT1364. (b) Effect of KCN, DNP, or azide on the % increase in Klett readings for *E. coli* cells carrying the Te^R plasmid pDT1364.

results suggest that electron transport is important to tellurite resistance.

3.25. Anaerobic growth in the presence of tellurite.

E. coli JM83 strains carrying pUC8, pDT1364 or pDT1558 which had been grown under anaerobic conditions in LB or in minimal media containing glucose but no electron acceptor, were also able to rapidly reduce tellurite to tellurium. This indicated that the enzymes necessary for this reduction were present even in anaerobically grown bacteria. In addition, *E. coli* MI1443, which has a deletion in fumarate reductase, and *E. coli* DSS106, which has a deletion in DMSO reductase, were tested for their ability to reduce tellurite to tellurium. These strains were grown under anaerobic conditions in media containing glycerol and either fumarate (DSS106) or DMSO (MI1443). Upon addition of tellurite, both cultures immediately began to turn black indicating that DMSO reductase and fumarate reductase are not necessary for this reaction.

Some bacteria that are resistant to selenite are able to use selenite as an electron acceptor under anaerobic conditions (Macy and Rech, 1990; Steinberg et al., 1990). The possibility that the Te^R determinants may allow *E. coli* to use potassium tellurite as an electron acceptor under anaerobic conditions was investigated. Bacterial strains carrying pUC8, pDT1364 or pDT1558 were inoculated into media

containing glycerol, a nonfermentable growth substrate, and either potassium tellurite or fumarate as an electron acceptor and incubated for 2 days at 37°C in sealed anaerobic flasks. Very little growth was observed in the flasks containing only tellurite as an electron acceptor suggesting that it cannot be used as the sole electron acceptor under the conditions described here.

4. Discussion

This study represents the first attempt to compare two plasmid-mediated tellurite resistance (Te^R) determinants and examine their encoded mechanisms of resistance. The Te^R plasmids RK2 (also called RP4 or RP1) and pHH1508a were chosen as representatives of the incompatibility groups P and H, respectively, because these two incompatibility groups are most commonly found to carry this type of metal resistance. The plasmid RK2 has been extensively studied because of its early identification in bacteria from clinical isolates and because of its broad host range. The Te^R determinant on this plasmid was not identified until recently because of its silent phenotype on most plasmid isolates. Much less is known about the plasmid pHH1508a, however, it may also be important in mediating antibiotic resistance in pathogenic bacteria.

A complete genetic analysis of the tellurite resistance regions of these plasmids was undertaken. The recent study of the Te^R determinant carried by the IncHI2 plasmid pMER610 from a species of *Alcaligenes* made it possible to compare our results with a third tellurite resistance determinant (Jobling and Ritchie, 1987, 1988).

Expression of tellurite resistance. Expression of the Te^R determinant of pMER610 appears to be inducible by prior exposure to low levels of potassium tellurite as shown by

growth challenge experiments. The same method was used to study expression of the Te^R determinant of pHH1508a. Upon challenge with a high level of tellurite, there was no difference in the growth rate of cells which had been previously exposed to low sub-toxic levels of tellurite and those which had not been exposed. Therefore, it appears that this Te^R determinant is expressed constitutively.

The plasmid RP4Te^R does not normally express Te^R, but when plated on tellurite media, plasmid variants can be obtained which appear to be identical to the original plasmid except for the ability to express tellurite resistance (Taylor and Bradley, 1987). The results presented here suggest that this Te^R determinant, once turned on, is also expressed constitutively. The absence of regulation in the Te^R determinants of RP4Te^R and pHH1508a suggests that these determinants are unrelated to the IncHI2 determinant.

The mechanism of regulation of the Te^R determinant of pMER610 has not yet been determined. Recent studies using mini-Mu transcriptional and translational fusions suggest that the IncHI2 Te^R genes themselves are constitutively expressed (Jobling and Ritchie, 1988). Therefore, the induction of resistance in growth challenge experiments may be related to the host cell response in this system.

Cloning of the Te^R determinants. The Te^R determinants of both RK2Te^R and pHH1508a were cloned into the vector pUC8 for genetic analysis. This plasmid was used as a vector since it

contains a variety of restriction endonuclease sites in its multiple cloning region and because of the relative ease with which recombinant clones can be selected by testing for loss of expression of β-galactosidase. In addition, sequencing of DNA inserts can be directly performed using the "universal" forward and reverse primers.

The restriction endonuclease maps of the Te^R clones pDT1364 and pDT1558, derived from pHH1508a and RK2Te^R respectively, showed no similarity to each other or to that of the Te^R determinant of pMER610. This suggested that either the determinants were not related to each other or that they had undergone sufficient evolutionary divergence to have lost all similarity in their restriction patterns. Since a small number of mutations could have accounted for the observed difference in the restriction maps, the technique of DNA-DNA hybridization was used in order to determine if there was any homology between the Te^R determinants from pHH1508a and RK2. Under conditions of low stringency (37°C, 50 % formamide), no homology could be detected suggesting that these two determinants are unrelated.

The minimum inhibitory concentration (MIC) of tellurite for bacteria carrying the Te^R clones was compared with that for bacteria carrying the parent plasmids. The level of Te^R encoded by the clone pDT1364 is four-fold lower than that of pHH1508a. This could be due to its expression on the high copy number plasmid, pUC8. Resistance to tetracycline (Tet^R)

decreases when the determinant is present at a high copy number in the cell apparently due to the effect of large amounts of the Tet^R protein in the inner membrane (Moyed et al., 1983). Similarly, this could account for the slightly lower level of resistance of the clones pDT1366 and pDT1558 compared to the original plasmid RP4 which has a copy number in *E. coli* of only about 4-7 per chromosome (Figurski et al., 1979). Both pHH1508a and RK2Te^R increase the MIC of *E. coli* by about 1000-fold. In contrast, a chromosomal mutation to Te^R only confers about a 20-fold increase in resistance (Tomás and Kay, 1986).

Localization of the Te^R genes. The Te^R genes were localized on the pUC8 clones using the technique of transposon insertion mutagenesis. Tn*1000* was used because it transposes randomly at fairly high frequencies and its DNA sequence is known (Guyer, 1983). Random insertions of Tn*1000* into each Te^R determinant were isolated and screened for loss of Te^R. By restriction mapping of twenty of these insertions, the Te^R determinant on pDT1364 was localized to a minimal 1.25 kb region. Because of the inherent error involved in restriction mapping, the possibility of nonrandom insertion of the transposon, and the fact that only transposon insertions which eliminated Te^R were mapped, this size was only a rough estimate.

Transposon mutagenesis of the Te^R determinant of RK2Te^R indicated that it spans a minimum of 1.75 kb of DNA (not

including *kilA*, to be discussed below). This is close to the 1.9 kb size determined by Taylor and Bradley (1987) using Tn7 insertion mutagenesis. These size estimates for the HII and Pα Te^R determinants are much smaller than the minimum 3.55 kb size determined for the Te^R determinant of pMER610 using the same technique (Jobling and Ritchie, 1987). This again suggested that the Te^R determinants of RK2Te^R and pHH1508a are unrelated to that of pMER610, and probably unrelated to each other as well.

Tn*1000* insertions into the Te^R region of pMER610 prepared by Jobling and Ritchie (1987) could be divided into two groups: those that caused complete loss of resistance to tellurite, probably because of a structural mutation, and those which resulted in only partial loss of resistance to tellurite, probably due to a mutation in the regulatory region. In this study, Tn*1000* insertions into the Te^R region of pDT1364 all resulted in a lowering of the minimum inhibitory concentration of potassium tellurite down to the level of plasmid-free *E. coli*. This is consistent with the presence of only structural genes and no regulatory regions.

In contrast, while most Tn*1000* insertions into pDT1558 resulted in a slightly lower level of resistance than the plasmid-free *E. coli*, one mutant, which was located at the junction between the *kilA* gene and Te^R region, had an intermediate level of resistance. Since expression of tellurite resistance by this Te^R determinant appears to be constitutive, the lower level of activity does not appear to

be due to insertion in a regulatory gene as hypothesized for the hyposensitive insertions in the pMER610 determinant (Jobling and Ritchie, 1987). The reduced level of resistance could have been due to the insertion of Tn*1000* near the end of a Te^R gene, creating a truncated protein with partial activity. Alternatively, Tn*1000* could have inserted into the promoter region of the Te^R gene(s), resulting in a lower level of gene expression and, therefore, a lower level of resistance. Nucleotide sequencing of this mutant (discussed below) indicated that Tn*1000* had inserted into the end codon of the *kilA* gene and that the loss of resistance was probably due to interference with the normal transcription of the Te^R genes. A low level of transcription of the Te^R genes could be occurring from a promoter within Tn*1000* which has outreading promoters located at both ends (Lers et al., 1989).

Phenotypes associated with Te^R. Recent studies on the fertility inhibition (*fiw*) properties of RP1 suggested that one of the functions (*fiwB*) which can reduce the transfer of IncW plasmids such as R388 was located in the Te^R region (Fong and Stanisich, 1989). The experiments presented here were unable to show the expression of fertility inhibition by the Te^R clones constructed. The possibility remains that the *fiwB* determinant overlaps the Te^R region but the entire region necessary for the expression of this property may not have been cloned.

Early studies on Te^R specified by IncH plasmids showed an association between resistance to tellurium compounds and the ability to inhibit the development of coliphages such as bacteriophage λ (Taylor and Summers, 1979). Although the ability of *E. coli* strains carrying pHH1508a and pDT1178 to support the development of the phage λ is decreased at least 10^6 times compared to a plasmid-free strain of *E. coli*, the presence of the clone pDT1364 did not result in phage inhibition (*phi*). This suggests that the Te^R and *phi* phenotypes are not functionally associated.

Expression of Te^R proteins. The widely different size estimates for the Te^R determinants on the plasmids RK2Te^R, pHH1508a, and pMER610 immediately suggested large differences in the sizes of the proteins that could be produced. This was supported by expression of the Te^R determinants using *in vitro* transcription-translation. pDT1364 was found to express two polypeptides of 23 kDa and 12 kDa which were not encoded by pUC8. The observation that some of the Te^S Tn*1000* insertion mutants of pDT1364 did not express the 23 kDa polypeptide indicated that this protein is involved in resistance to tellurite. One mutant, which had Tn*1000* inserted in the middle of the Te^R determinant, expressed a small unique polypeptide of about 9.5 kDa which appeared to be a truncated form of the 23 kDa Te^R protein. This suggested that transcription of the Te^R genes was occurring from right to left on the map of pDT1364 (Figure 5b).

Unfortunately, examination of the other Tn*1000* insertion mutants did not reveal any pattern of truncated polypeptides which could be correlated with the site of insertion of Tn*1000* as observed by Taylor et al., (1987) with Tn*1000* insertion mutants of the *tetO* determinant of *Campylobacter jejuni*. The observation that none of the Tn*1000* insertion mutants showed any change in the 12 kDa polypeptide suggested that this polypeptide is not involved in tellurite resistance.

In vitro transcription-translation showed that pDT1558 encoded a polypeptide of 40 kDa which was not expressed by pUC8 and is probably involved in tellurite resistance. The expression of a protein of approximately 40 kDa by transposon Tn*1000* made it difficult to examine Tn*1000* insertion mutants of pDT1558 for lack of expression of the Te^R protein. However, two of the insertion mutants examined in this system produced polypeptides which were smaller than the putative Te^R protein and therefore might represent truncated mutants of this protein. The sizes of these proteins suggested that the 40 kDa Te^R protein is transcribed from right to left on the map of pDT1558 (Figure 4b).

In vitro transcription-translation is a useful technique for the identification of plasmid-encoded polypeptides (Dougan and Fairweather, 1988). However, it can be misleading since not all of the Te^R proteins could be detected using this system as experiments using the T7 RNA

polymerase/promoter overexpression system have demonstrated here (discussed below).

Transcriptional and translational analysis of the IncPα Te^R determinant. The Te^R determinants were studied further by preparing transcriptional and translational fusions to the gene for β-galactosidase (*lacZ*) using the transpositional phages mini-MudI and mini-MudII respectively. Since only insertions which resulted in expression of β-galactosidase were studied, it is not apparent whether transposition of mini-Mu was random or if there were hot spots for insertion.

Transcription and translation of the IncPα Te^R genes was shown to be in the same direction as *kilA*. Furthermore, a large number of fusions with the *kilA* gene were obtained which eliminated or significantly reduced the level of Te^R. This suggested that either the Te^R genes were being transcribed from the *kilA* promoter or the *kilA* gene is required for full expression of tellurite resistance.

Relationship between Te^R and plasmid replication control genes on RK2. The relationship between Te^R and *kilA* was investigated further by determining the nucleotide sequence of the Te^R region of RK2Te^R. Two open reading frames, *telA* and *telB*, in addition to *kilA* were discovered. There was no homology to the *E.coli* consensus promoter in the region upstream of the *telA* gene; therefore, RNA primer

extension was used to determine the site of initiation of transcription of these genes. Using an oligonucleotide which was complementary to the 5' end of the *telA* gene, AMV reverse transcriptase was used to extend the mRNA. Termination of primer extension occurred at a site within the *kilA* gene about 600 bases upstream of the primer; however, there were no promoter-like sequences in this region and insertions of Tn*1000* and mini-Mu upstream of this site eliminated tellurite resistance. Termination of transcription in this experiment may have been due to secondary structure in the mRNA. Alternatively, the 5' end of the mRNA transcript may have been processed *in vivo*.

When a primer complementary to a sequence within *kilA* was used, RNA primer extension indicated that transcription initiation occurred 7 bases downstream of the -10 region previously identified for the *kilA* gene. This site has many similarities to other transcription initiation sites. Based on the results obtained using primer extension and *lac* fusions, it appears that the *telA* and *telB* genes are transcribed from the same promoter as *kilA*.

The *kilA* gene is negatively regulated by the *korA* and *korB* gene products and by *korE* (Young et al., 1985; Young et al., 1987). If *kilA* and *telAB* do form an operon, *telA* and *telB* should be regulated by *korA* and *korB* in the same manner as *kilA*. A palindrome has been found within the *kilA* promoter that overlaps the -10 sequence and which is "necessary and sufficient" to allow regulation by KorA (Young

et al., 1985) Another palindrome, near the -35 sequence is the putative target for the corepressor KorB (Smith et al., 1984; Young et al., 1987). *korE*, located upstream of *kilA*, is a trans-acting function which participates in the control of *kilA* and inhibits *kilA* at the level of gene expression (Young et al., 1987). *korE* might bind to the *korA* product to increase its affinity for the *kilA* promoter or it might stimulate *korA* expression.

The suggestion that the *telA* and *telB* genes are regulated by KorA and KorB does not contradict earlier experiments suggesting that Te^R is constitutive. These experiments indicated that Te^R was not induced by the presence of tellurite and that cells expressed Te^R immediately upon addition of tellurite. The normal regulated level of transcription from the *kilA* promoter is probably very low but may be sufficient for expression of Te^R. The *lacZ* fusions described in this study will be useful in the testing of this hypothesis since *lacZ* fusions have been used extensively in the study of transcriptional and translational regulation of genes (Silhavy et al., 1984).

The relationship of Te^R to the plasmid replication control genes including *kilA*, *korA*, and *korB*, and why they all appear to be encoded together on a transposon (Bradley and Taylor, 1987) is still unclear. The *telA* and *telB* genes may have some other function, for example in plasmid replication control, which has not yet been identified. No transposase which could function in the transposition of this region has

been found. The existence of a transposon in this region is supported by observations that spontaneous deletions can occur in this region of RP4 (Sakanyan et al., 1978).

The product of the *kilA* gene was predicted to be a 28,391 dalton polypeptide. The KilA protein was overproduced using the T7 RNA polymerase/promoter system. The apparent molecular weight on SDS-PAGE of 31 kDa corresponded well to the predicted size. The isolation of a large number of lac$^+$ transcriptional and translational fusions of *kilA* to *lacZ* indicated that transcription and translation of this gene was occurring. In addition, it suggested that KilA is located in the cytoplasm since the activity of β-galactosidase is lost if the cell attempts to export it (Bassford et al., 1979; Silhavy and Beckwith, 1985; Manoil, 1990). This appears to be due to the presence of stop-transfer sequences. This location for KilA was also supported by hydrophobicity plot analysis of the predicted amino acid sequence.

The formation of β-galactosidase fusions to study membrane protein topology has been used to complement fusions to alkaline phosphatase (Froshauer et al., 1988; Herrero et al., 1988). In this way, both periplasmic and cytoplasmic domains can be identified. However, LacZ fusions appear to be less reliable in this regard (Georgiou et al., 1988). β-galactosidase activity is sometimes high even when fused to a region shown by other methods to be located in the periplasm. In some cases, this is because the β-galactosidase moiety can prevent membrane insertion segments from functioning

properly. In other cases, it appears that the hybrid protein can be cleaved by proteolytic enzymes in the cell, releasing the active β-galactosidase into the cytoplasm (Georgiou et al., 1988). Thus, evidence other than *lac* fusions is necessary to determine unambiguously the location and possible membrane topology of these proteins.

The results of the transcriptional and translational analysis suggest that either expression of *kilA* is not lethal to the cell under all circumstances or that the host cell has developed some resistance to its function (Young et al., 1985). This function is not yet known. Several other cell lethal genes have been found to be encoded on plasmids. These include: the *ccd* genes of the F factor, which appear to be involved in coupling cell division to plasmid replication (Ogura and Hiraga, 1983; Jaffé et al., 1985); the *hok/sok* genes of plasmid R1 (Gerdes et al., 1986); the *kil* and *kor*-like genes of pKM101, a derivative of the IncN plasmid R46 (Winans and Walker, 1985); and the *kil* genes on pIJ101 in *Streptomyces*, which are necessary for plasmid transfer (Kendall and Cohen, 1987; Stein et al., 1989). Recently, the expression of *kilA* was suggested to cause *Pseudomonas aeruginosa* cells to form long filaments (Cuskey et al., 1990). Further work will be required to determine if KilA affects cell division in *E. coli*. The overproduction of this protein described here will enable the study of its function and possible roles in either RK2 plasmid host range

or plasmid replication control (Barth et al., 1984; Schreiner et al., 1985; Thomas and Smith, 1987).

Analysis of TelA and TelB. The isolation of several translational fusions of *lacZ* to the *telA* gene and the absence of any long stretches of hydrophobic residues in its sequence suggests that TelA is a cytoplasmic protein. The product of *telA* was predicted to be a 42,130 dalton protein. This corresponds well to the apparent molecular weight of 40 kDa estimated by SDS-PAGE analysis of the *in vitro* transcription-translation products. However, when this protein was overproduced using the T7 RNA polymerase/promoter system, methionine-free media, ^{35}S-methionine and rifampicin to inhibit *E. coli* RNA polymerase, it had an estimated molecular weight of 48 kDa when examined by SDS-PAGE. This suggested that the smaller size observed using *in vitro* transcription-translation was due to premature termination of translation or lack of post-translational modification *in vitro*. However, further work showed that when this protein was overproduced by *E. coli* induced in Luria broth in the absence of rifampicin, a 42 kDa protein was again observed. The reason for this variation in molecular weight is not clear. Changes in apparent molecular weight in SDS-PAGE are sometimes caused by changes in the salt concentration. However, the migration of the IncHII Te^R protein, TehB, was not affected. Further research is

required to determine if TelA is phosphorylated or acquires a prosthetic group or cofactor *in vivo*.

The *telB* gene, however, appears to code for a 32,375 dalton membrane-spanning polypeptide based on the hydrophobicity profile. Isolation of active gene fusions to alkaline phosphatase and β-galactosidase using Tn*phoA* and mini-Mu, respectively, suggests that this protein is located in the inner membrane with portions extending into the periplasm and cytoplasm. This is based on the assumption that fusion to PhoA does not alter the topology or membrane insertion of TelA.

The active form of alkaline phosphatase is a zinc-containing dimer. This conformation is only possible in the high pH, non-reducing environment of the periplasm (Schlesinger et al., 1969; Boyd et al., 1987). Thus, active fusions to PhoA are only possible when it is fused to a periplasmic facing portion of a protein (Manoil, 1990). Gene fusions to alkaline phosphatase have been used extensively to determine the topology of both inner and outer membrane proteins. The topology of the inner membrane protein leader peptidase determined using PhoA fusions was shown to be consistent with the topology proposed on the basis of proteolytic treatment of membrane preparations (San Millan et al., 1989). Weak PhoA activity, however, can be obtained with fusions to cytoplasmic domains and even fusions which are out of frame (Akiyama and Ito, 1987; Gött and Boos, 1988). In this study, both of the *phoA* fusions that were

sequenced produced very blue colonies on XP plates and therefore were considered to be of high activity; however, the level of alkaline phosphatase activity was not measured. The fusions were found to be in the correct reading frame for production of TelB'-'PhoA and TehA'-'PhoA fusion polypeptides. Recently, the periplasmic enzyme β-lactamase which confers resistance to ampicillin has also been used in the construction of protein fusions and the study of membrane protein topology in the same manner as alkaline phosphatase (Broome-Smith and Spratt, 1986; Forst et al., 1987).

A potential ORF was identified on the opposite strand of the DNA from the *telA* and *telB* genes. This ORF could potentially code for a 35 kDa protein. The CAI value for this ORF was 0.295 which falls within the range for *E. coli* genes (Sharp and Li, 1987a) and is higher than the CAI value for some of the Te^R genes studied here. High CAI values, reflecting greater codon bias, have been found for highly expressed genes such as the ribosomal protein genes, *rpsU*, and *rpoD*, which have CAI values of 0.726 and 0.582, respectively. Genes coding for poorly expressed proteins have low CAI values. Examples include the regulatory gene, *trpR*, which has a CAI value of 0.267, and the restriction and modification specificity gene, *hsdS*, which has a CAI value of 0.218 (Gough and Murray, 1983; Sharp and Li, 1987a). Furthermore, plasmid and transposon genes from *E. coli* appear to have less codon bias than *E. coli* chromosomal genes (Gouy and Gautier, 1982). Thus, it is possible that this ORF is

translated and perhaps it even encodes the *fiwB* determinant identified by Fong and Stanisich (1989). However, since no lac$^+$ transcriptional or translational fusions of mini-Mu were obtained in this direction and no promoter or ribosome binding sites could be found upstream of this ORF, this remains simply a speculation.

A search of protein and DNA sequences in current data banks did not reveal any proteins with a significant level of homology to KilA, TelA, or TelB throughout their length. Some examples of proteins which had short regions of similarity were shown. However, the significance of these is questionable because of the low percent homology, the short regions of the proteins involved, and the number of spaces which had to be added for optimal alignment. For example, the significance of a 50 aa region in TelB having 49 % homology with part of Aleurain, a barley thiol protease, is difficult to imagine. Some similarities were found between TelB and colicin A from *Citrobacter freundii* and also the proline carrier protein from *E. coli*. These similarities were mainly in hydrophobic domains and may be examples of convergent evolution. Further work on the functions of these regions in TelB will be required before the significance of these similarities can be understood.

It does appear to be significant, however, that some similarity was found between the Cys..Cys region of TelB and the cation binding regions of glutathione reductase, and mercury and cadmium binding proteins. It remains to be seen,

however, if this Cys..Cys region of TelB is involved in binding either tellurite or reducing equivalents. In the proposed model of TelB, this region is located in the cytoplasm. This would be an appropriate location for either of these functions.

Difference between RK2 and RK2Te^R. The difference between RK2 and its tellurite-resistant variant RK2Te^R was investigated by comparing their DNA sequences. Two frame-shift mutations were found which could be responsible for this difference. In addition, a missense mutation was found which results in a serine to cysteine change at amino acid residue 125. The importance of this residue in, for example, binding to tellurite, should be studied. Oligonucleotide site-directed mutagenesis of the Cys residues in TelB will be useful in this regard.

Te^R variants of RP4 are readily generated upon plating on tellurite media (Bradley, 1985). This is most consistent with a small number of mutations such as were found here. The plasmids RP4, RK2, RP1, R18, and R68 were all isolated from the same hospital in Birmingham in 1969 and may be different isolates of the same plasmid. Loss of expression of Te^R by this plasmid may have occurred either before isolation of the plasmid or during subsequent laboratory subculturing. Further studies are required to determine if all RK2 plasmids have identical mutations in their Te^R

determinants. Sequencing of a number of pairs of Te^S and Te^R RK2 plasmids is currently being attempted.

IncP plasmids are useful in genetic manipulation of many gram-negative bacteria because of their broad host range. The ability of Te^R to be expressed in many bacteria (Bradley et al., 1988) will make this a useful resistance marker for the construction of cloning vectors. If it can be shown that it is not important in virulence, it could also be useful as a selective marker in bacterial pathogens and bacterial vaccine constructs in which it would be imprudent to introduce antibiotic resistance determinants.

Sequencing analysis of the IncHII Te^R determinant. The direction of transcription and translation of the IncHII Te^R genes was confirmed to be from right to left on the map of pDT1364 through the study of mini-Mu *lacZ* fusions (Figure 5b). Nucleotide sequencing of the IncHII Te^R determinant revealed the presence of two open reading frames which have been called *tehA* and *tehB*.

The product of the *tehA* gene has a predicted molecular weight of 35,908 daltons based on the largest open reading frame in this region preceded by a potential Shine-Dalgarno sequence. The exact start site of this ORF is unclear since there are several potential start codons in this region. A functional fusion with Tn*phoA* was obtained in amino acid residue 43, and, therefore, the actual N-terminus must be

very close to that shown here in order for there to be enough room to encode a membrane-spanning domain.

A possible model for this polypeptide was proposed which suggests the presence of a large number of transmembrane segments. Conflicting results were obtained with the two methods for prediction of transmembrane domains. The method of Eisenberg et al., (1984b) suggested 10 membrane helices, whereas the method of Kyte and Doolittle (1982) only identified 5. Further work will be required to confirm the location of the periplasmic and cytoplasmic domains. This protein thus provides an excellent opportunity to test the value of these two prediction algorithms.

The highly hydrophobic nature of TehA could account for the smaller than expected molecular mass (28 kDa) of the protein after overexpression in the T7 RNA polymerase/promoter system, since hydrophobic membrane proteins often show abnormally fast migration on SDS-PAGE gels (Chopra, 1986).

The second gene in this determinant, *tehB*, appears to encode a relatively hydrophilic polypeptide with a predicted molecular weight of 22,516 daltons. This corresponds to the 23 kDa protein seen by SDS-PAGE of both *in vitro* transcription-translation products and cell lysates containing overproduced Te^R proteins. This polypeptide is probably located in the cytoplasm since fusions to LacZ but not PhoA were obtained. Further work involving separation of

cytoplasmic and membrane cell fractions will be required to confirm this location.

Initial studies using *in vitro* transcription-translation suggested that a single protein of about 23 kDa was encoded by the Te^R region of pHH1508a. The second protein of about 36 kDa was only detected by overproduction and labelling with L-[^{35}S]methionine using a T7 RNA polymerase/promoter system. This demonstrates the usefulness of this system. The RNA polymerase from bacteriophage T7 is specific for a large promoter sequence which is different from that of *E. coli*. Promoters which can be utilised by T7 RNA polymerase have not been found in DNA unrelated to bacteriophage T7 (Chamberlin et al., 1970). In addition, unlike *E. coli* RNA polymerase, T7 RNA polymerase is resistant to the effects of rifampicin. The T7 overexpression system used here takes advantage of these properties, allowing the selective production of large amounts of specific mRNAs and proteins in a very short time (Tabor and Richardson, 1985).

Both TehA and TehB have a similar percentage of methionine residues in their sequences but much more of the 23 kDa TehB polypeptide is seen on the autoradiogram. This could be caused by the highly hydrophobic TehA protein not entering the SDS-PAGE gel, since bands were consistently seen at the beginning of both the stacking and separating gels. Alternatively, this could be caused by low translational efficiency of *tehA* due to the poor SD sequence in front of this gene.

Poor expression of the *arsB* gene in the arsenical resistance operon has been found to be due to differential mRNA stability (Owolabi and Rosen, 1990). ArsB is a highly hydrophobic polypeptide located in the inner membrane and is a subunit of an arsenite-translocating ATPase (San Francisco et al., 1989; Chen et al., 1986). In this system, there is selective degradation of the *arsB* segment of the single polycistronic mRNA synthesized. Furthermore, it was observed that differential expression is more noticeable with integral membrane proteins. Thus, differential mRNA stability should also be investigated as a possible cause of lower expression of the TehA polypeptide.

Another possible explanation for the much higher level of expression of *tehB* could be the presence of an intercistronic sequence which enhances translational initiation. This has been found to account for increased expression of the gene for the c subunit of ATP synthase from *E. coli* (McCarthy et al., 1985). In contrast, the rate of translation of the gene for the ε subunit of the ATPase has been shown to be reduced by the presence of secondary structure in the mRNA upstream of the coding sequence (Dunn and Dallmann, 1990). These possibilities should also be examined.

Differential expression could mean that TehA and TehB are not in a 1:1 ratio within the bacterial cell or that expression of TehA limits the expression of tellurite resistance. Codon usage values determined for these two

genes also suggested a lower level of expression of *tehA* compared to *tehB*.

The HII Te^R DNA sequence was also searched for the presence of other possible functional domains such as ATP-binding sites, DNA-binding sites, and metal-binding sites; however, no significant homology to the consensus sequences for these domains was found. Further work will be required to determine if nucleotide binding sites are present, for example, by using photoaffinity labeling with ATP (Yue and Schimmel, 1977).

Prediction of membrane protein topology. Possible models for the putative membrane proteins TelB and TehA were proposed (Figure 30; Figure 40). Membrane-spanning regions were predicted from the sequences using the algorithms of Kyte and Doolittle (1982) and Eisenberg et al., (1984b). These methods involve searching for long segments in the amino acid sequence which are highly hydrophobic. This is based on the assumption that the transmembrane domains are α-helices. In this form, 21 amino acids would be required to span the nonpolar part of the membrane (von Heijne, 1985b). However, membrane-spanning α-helices may not need to be highly hydrophobic if they interact with other α-helices within the membrane (Eisenberg et al., 1984b). Furthermore, simply searching for long highly hydrophobic sequences can be misleading since "one of the most hydrophobic sequences known

is a stretch of 19 residues in dogfish lactate dehydrogenase, a water-soluble protein" (Jennings, 1989).

Because of the difficulty in crystallizing these hydrophobic proteins, only a handful of membrane proteins have had their 3-dimensional structures determined (Lloyd and Kadner, 1990). One of the first membrane proteins to have its tertiary structure determined was bacteriorhodopsin, a protein found in the purple membrane of *Halobacterium halobium* (Henderson and Unwin, 1975). Several of its membrane-spanning regions were shown to be α-helices. However, other proteins such as the outer membrane protein, porin, in *Escherichia coli* have transmembrane domains which form β-sheet structure (Paul and Rosenbusch, 1985; Kleffel et al., 1985). Membrane-spanning regions in β-sheet structure may require as few as 6 aa to span the membrane and are therefore more difficult to detect in the sequence (Eisenberg, 1984; Ferenci, 1989).

Even the determination of the hydrophobicity of individual amino acids is controversial. The method of Kyte and Doolittle (1982) is one of the most commonly used methods and may be more reliable than many others because their hydrophobicity scale takes into account several different factors (Fasman, 1989). These include the free energy of transfer of the side chain of the aa between water and vapor (and thus the preference of the amino acid for hydrophilic and hydrophobic phases) and the distribution of amino acids between the interior and exterior of a protein. However,

they also state that "we did not hesitate to adjust the values subjectively". In this study, the overall hydrophobicity of the Te^R proteins was estimated both by plotting the average hydrophobicity of a window of 11 amino acid residues (the hydrophobicity plot) and also by counting the total number of seven amino acids (IFLVMAT) which had relatively high hydropathy indices according to Kyte and Doolittle (1982). Since different authors arrive at different scales of hydropathy and may even consider some of these amino acids to be hydrophilic rather than hydrophobic, the percentage hydrophobic residues listed here can only be used for comparison within this study.

These models for the membrane proteins TelB and TehA were based solely on the sequence data and one site of insertion of Tn*phoA* in each polypeptide. Therefore, they can only be used as a starting point for further study of the structure of these polypeptides. The isolation of active Tn*phoA* insertions into each of the putative periplasmic domains will help to confirm these conformations. This may not be possible if, for example, PhoA fused to other periplasmic domains is unable to form a dimer for other reasons such as steric interference from other parts of TelB. Alternatively, since the sites of insertion of Tn*phoA* identified here appear to be hot spots, the likelihood of isolating insertions elsewhere within these two genes may be very low. More traditional methods such as sensitivity to proteolysis may be necessary to determine its topology.

Comparison of the three Te^R determinants. No similarity could be found between the sequences of the two Te^R determinants from RK2 and pHH1508a or with the sequence of the Te^R region of the IncHI2 plasmid pMER610 (Jobling and Ritchie, 1988). However, each Te^R determinant appears to encode a membrane protein of similar size (TelB [Pα], 32.4 kDa; TehA [HII], 36.5 kDa; ORF3 [HI2], 38.3 kDa). These may have some functional similarity. However, they appear to be significantly different based on analysis of their amino acid sequences, having different numbers of predicted transmembrane domains and different proportions of acidic, basic and Cys residues. The presence of only two proteins in the P and HII Te^R determinants suggests a much simpler mechanism for resistance mediated by these two plasmids compared to the four or five proteins encoded by the HI2 Te^R determinant.

The presence of at least three different plasmid-encoded Te^R determinants with no sequence homology suggests that the evolution of Te^R may occur quite readily and that this resistance is important to bacteria either in the environment or in colonization of the human body.

Electron microscopy of Te^R bacteria. Through the use of electron spectroscopic imaging of bacteria carrying the Te^R clones described here, Taylor et al (1988) confirmed earlier reports from X-ray diffraction analysis that bacteria grown

in the presence of potassium tellurite contain metallic tellurium and showed that the black crystals visualized within the cells are composed of metallic tellurium. This suggested that resistance may be due to reduction of the toxic K_2TeO_3 to less harmful metallic tellurium, possibly by a plasmid-encoded "tellurite reductase". This mechanism was supported by the observation that much more electron dense tellurium metal is found in bacterial cells carrying a large number of copies of the Te^R determinant (pDT1364, pDT1366) than in bacterial cells carrying few copies of the Te^R determinant (pHH1508a, RK2Te^R).

Tellurium crystals were frequently observed just inside the inner membrane of the bacterial cell. This suggested that proteins in the inner membrane may be important in the transformation of tellurite to tellurium. This contrasts with studies on the reduction of selenite to elemental selenium. *E. coli* cells grown in the presence of sodium selenite form irregular deposits of selenium which are located within the cytoplasm but are not associated with the cell membrane (Silverberg et al., 1976).

Another possible mechanism of resistance is the presence of a specific plasmid-encoded transport system which pumps K_2TeO_3 out of the cells. Resistance to tetracycline (McMurry et al., 1980), cadmium ions (Tynecka et al., 1981b), and arsenate ions (Mobley and Rosen, 1982) have all been shown to be mediated by mechanisms which depend on active efflux of the toxic substance. Such a transport system could be

located in the inner membrane. The localization of the Te^R proteins in the inner membrane could explain the reduction in the MIC for tellurite when the number of copies of the Te^R determinant is increased since this phenomenon also occurs with tetracycline resistance (Moyed et al., 1983).

Studies on the mechanism of Te^R. Each of the Te^R determinants may encode one of several different possible mechanisms of resistance, including reduced uptake, increased efflux or detoxification of the toxic tellurite. A preliminary investigation of the mechanisms of resistance to tellurite mediated by the plasmids pHH1508a and RK2Te^R was undertaken. Media-conditioning experiments were performed in an attempt to differentiate between these different possible mechanisms: if the Te^R plasmids encode a tellurite reductase, they may accumulate the tellurium metal inside the bacterial cells and thereby decrease the concentration of toxic K_2TeO_3 around them, thus making it possible for tellurite-sensitive bacteria to grow; alternatively, if either an efflux or reduced uptake mechanism was encoded, the concentration of toxic tellurite in the medium around the bacteria should not be reduced.

Media-conditioning experiments on agar could not detect any significant difference in the ability of *E. coli* JM83 carrying pUC8, pDT1558, or pDT1364 to inactivate tellurite. However, a significant difference was seen in media-conditioning experiments using tellurite broth. Bacteria

carrying pDT1364 reduced the toxicity of tellurite broth by at least 64-fold, suggesting that this plasmid encodes a tellurite-detoxification system.

In contrast, bacteria carrying pDT1558 only reduced the toxicity of tellurite broth by half, suggesting that a different mechanism is encoded. Therefore, the possibility that the *tel* genes code for reduced uptake or increased efflux of tellurite should be considered. Measurement of the total amount of tellurium compounds taken up into the cells can be measured using a radioactive isotope of tellurium (Cooper and Few, 1952). In addition, however, the possibility that the lack of growth by *E. coli* JM83 in less diluted filtrate is due to the production of a toxic extracellular substance encoded by RP4 should also be tested.

Chromosomal resistance to tellurite is due to a mutation in one of the two phosphate transport systems (Tomás and Kay, 1986). Phosphate is transported into the bacterial cell by two systems (Rosenberg et al., 1977). Normally, phosphate is taken up by the *pit* (phosphate inorganic transport) system (Willsky and Malamy, 1974). Under conditions of low phosphate, the phosphate-specific transport system (*pst*) is induced, allowing scavenging of other sources of phosphate (Willsky et al., 1973). Perhaps the *tel* gene products affect the transport of tellurite by altering the rate of phosphate transport. Further studies must be done to determine the rates of phosphate transport in the presence and absence of these Te^R genes.

These proposed mechanisms of resistance, detoxification for HII Te^R and either reduced uptake or efflux for IncP, are consistent with previous observations that cells carrying the HII determinant contain much more black tellurium metal deposits than cells carrying the IncP determinant (Taylor et al., 1988).

The finding that no hypersensitive mutants were obtained during isolation of insertions of Tn*1000*, Tn*phoA* or mini-Mu suggests that the IncPα and IncHII Te^R determinants do not encode tellurite-specific uptake systems, like the mercury resistance determinant, in spite of their putative membrane locations.

Reduction of tellurite. Preliminary experiments were performed to study the rate of reduction of tellurite to black metallic tellurium by Te^R and Te^S bacteria. Spectrophotometric methods have been used previously to measure the rate of reduction of tellurite to tellurium *in vitro* (Terai et al., 1958; Chiong et al., 1988b). Because of the very slow rate of this reaction *in vitro*, the rate was first studied in whole cells. Therefore, it was necessary to find an appropriate method of measuring the approximate rate of formation of black tellurium without interference due to light scattering by whole cells. When dilutions of bacteria containing black tellurium were measured using a Klett colorimeter, the values decreased proportionately. It was found to give reasonably satisfactory values which appeared

to reflect the extent of reduction of tellurite to tellurium as determined by visual inspection.

E. coli carrying the plasmid pDT1364 reduced tellurite to black tellurium at a much greater rate than bacterial cells carrying either pDT1558 or pUC8. This observation supports the hypothesis that this plasmid encodes a tellurite detoxification system. A greater extent of reduction of tellurite to tellurium by more resistant versus less resistant bacteria was noticed as long ago as 1966 with Streptococci (Tucker et al., 1966).

Cells overproducing the Te^R proteins did not have an increased rate of reduction of tellurite to tellurium. This could be due to segregation of the membrane proteins in intracellular inclusion bodies. Alternatively, it could be explained by a shortage of necessary cofactors in the bacterial cell.

The rate of reduction of tellurite was much slower when the cells were suspended in glucose buffer compared to buffer which contained no glucose. This is reminiscent of the glucose effect on systems controlled by catabolite repression (Magasanik and Neidhardt, 1987). Further work, including the use of various concentrations of glucose, should be carried out to examine this possibility.

The addition of potassium cyanide immediately eliminated reduction of tellurite by both Te^R and Te^S bacteria. Sodium azide also inhibited reduction of tellurite by *E. coli* (pDT1364). These compounds inhibit the transport of

electrons to oxygen by cytochrome oxidase (Alberts et al., 1983). Therefore, electron transport may be required for tellurite reduction. Alternatively, sodium azide or potassium cyanide may directly inhibit the putative tellurite reductase. In contrast, the *in vitro* activity of a tellurite reductase from *Mycobacterium avium* was activated by the presence of potassium cyanide (Terai et al., 1958). The mechanism of action of this enzyme is not yet understood.

The compound 2,4-dinitrophenol makes bacterial cells permeable to protons. The proton gradient is thus disrupted and the electron transport chain is accelerated in an attempt to compensate (Rosen, 1987). In this study, it was observed that DNP did not inhibit tellurite reduction to the same extent as KCN. This is consistent with the suggestion that the electron transport chain is important in tellurite reduction.

Three chromosomal mutants were tested during the course of this work. A mutant lacking thioredoxin, a potential electron donor, was still able to support the expression of Te^R by pDT1558 and pDT1364. This indicates that thioredoxin is not required for this reaction. However, another redox-active protein, glutaredoxin, can substitute for thioredoxin *in vivo*. These proteins can function in the reduction of sulfate to sulfite (Russel et al., 1990). Because of the chemical similarity between sulfur and tellurium, it may be useful to test recently constructed mutants lacking both

thioredoxin and glutaredoxin (Russel et al., 1990) for their ability to support tellurite resistance and/or reduction.

E. coli can rapidly reduce tellurite to tellurium after growth under both aerobic and anaerobic conditions. The enzymes responsible for this reaction may be identical in both situations. Alternatively, there may be two or more different reductases which are involved. Strains of *E. coli* lacking either DMSO reductase or fumarate reductase were both able to reduce tellurite to tellurium after growth under anaerobic conditions. This indicates that, despite the broad substrate specificity of DMSO reductase (Weiner et al., 1988), this enzyme is not required for tellurite reduction under anaerobic conditions.

These preliminary experiments on the mechanisms of tellurite resistance mediated by the plasmids RK2 and pHH1508a have raised many questions which need to be investigated. For example, the effect of the growth phase on reductase activity has not yet been determined and may significantly affect attempts to purify the putative "tellurite reductase". The possibility that the electron transport chain is involved in tellurite reduction needs to be rigorously studied. The Te^R proteins encoded by pHH1508a may directly reduce toxic potassium tellurite to metallic tellurium which has been shown to be deposited near the membrane. Alternatively, since both Te^R and Te^S *E. coli* are capable of tellurite reduction, these proteins may simply increase the rate of reduction by the host cell enzymes which

have not yet been identified. The testing of a library of *E. coli* chromosomal mutants with, for example, mutations in electron transport chain components, for loss of the ability to reduce tellurite or support tellurite resistance by these plasmids should prove useful in the identification of host cell proteins which are necessary for these phenotypes.

The mechanism of Te^R mediated by *Thermus thermophilus* strains appears to be due to a tellurite reductase which requires NADH or NADPH for activity (Chiong et al., 1988b). They were able to purify three different fractions having tellurite-reducing activity. One fraction contained two proteins having molecular masses of 51 and 53.6 kDa. The second fraction contained proteins of 53 and 55 kDa in size. A third fraction contained only one protein having a molecular mass of approximately 53 kDa (Chiong et al., 1988b). The sizes of the *T. thermophilus* proteins are significantiy larger than any of the plasmid-encoded Te^R proteins studied here.

Tellurite reductase activity from *Thermus* was still active after isoelectric focusing (Chiong et al., 1988b). However, no activity could be detected after electrophoresis under non-denaturing conditions of extracts of *E. coli* carrying the Te^R determinants of RK2 or pHH1508a. This negative result should not discourage similar experiments of this nature in the future since the biochemistry of tellurite reduction in *E. coli* is not yet understood. For example, there is no reason to expect the same electron donor, NADH,

to be used by tellurite reductases in both *E. coli* and *T. thermophilus*. In addition, even under non-denaturing conditions, separation of different components involved in tellurite reduction in *E. coli* may have occurred. Despite the expected differences in these two systems, it will be useful to continue to compare tellurite reductases from *Escherichia* and *Thermus* as these enzymes are characterized.

Tellurite resistance in the genus *Thermus* was not correlated with the presence of plasmids, therefore this resistance may be chromosomally-encoded (Chiong et al., 1988a). It will be interesting to see if these proteins are related to chromosomally-encoded proteins involved in tellurite reduction in *Escherichia coli* and other gram-negative and gram-positive bacteria. *Mycobacterium avium* extracts are also capable of reducing tellurite in the presence of NADH or malate and malic dehydrogenase but very little work has been done on this system (Terai et al., 1958). It is well known that gram-positive bacteria such as *Corynebacterium diphtheriae, Streptococcus faecalis,* and *Staphylococcus aureus* are commonly resistant to tellurium compounds but it is not known whether this resistance is chromosomally or plasmid-encoded or if the mechanism of resistance of these bacteria is related to those found in gram-negative bacteria.

Several species of bacteria, including a *Pseudomonas*, have been found to grow under anaerobic conditions using selenate as an electron acceptor (Macy and Rech, 1990;

Steinberg et al., 1990). In addition, *Enterobacter cloacae* may utilize toxic CrO_4^{2-} as an electron acceptor anaerobically; however, the biochemistry of this activity has not been studied (Wang et al., 1989, 1990). However, under the conditions studied here, *E. coli* cells carrying the Te^R determinants were unable to use tellurite as an electron acceptor under anaerobic conditions. Further experiments, including variation of the tellurite concentration used, should still be performed.

Conclusions. Resistance to potassium tellurite is mediated by many different plasmids found in both environmental and clinical isolates. This resistance may contribute to the selection of antibiotic resistance determinants found on the same plasmids.

An extensive genetic analysis of the tellurite resistance determinants of two of these plasmids, RK2 and pHH1508a, was undertaken. The results suggested that these two determinants are not related to each other or to the previously studied Te^R determinant of the plasmid pMER610 (Jobling and Ritchie, 1987, 1988). The existence of at least three different types of plasmid-encoded Te^R determinants in nature suggests that this resistance may be important to gram-negative bacteria in the environment or possibly in the colonization of the human body in which tellurium compounds appear to be relatively abundant (Schroeder et al., 1967).

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Appendix 1

Restriction Enzymes and Digest Conditions

Enzyme[1]	Abbreviation	Buffer[2]
*Acc*I	A	A
*Bam*HI	Ba	ABLM
*Bgl*I	Bg	H
*Bgl*II	Bl	M
*Bss*HII	Bs	A
*Dra*I	D	ABLMH
*Eco*RI	E	ABMH
*Eco*RV	EV	B
*Hinc*II	Hc	AMH
*Hind*III	Hd	BM
*Hpa*I	Hp	A
*Pst*I	Ps	H
*Sal*I	Sa	H
*Sma*I	Sm	A
*Sst*I	Ss	AH
*Sst*II	St	L
*Xba*I	Xb	H
*Xho*I	Xh	H

[1]Restriction endonucleases were obtained from Boehringer Mannheim Biochemicals, Montreal, Quebec, or Bethesda Research Laboratories (BRL) Canada, Ltd., Missauga, Ontario.

[2]Restriction endonuclease digestions were carried out using Boehringer Mannheim SuRE/Cut buffers.

Appendix 2.

Amino Acid Abbreviations and Hydrophobicity Values

Amino Acid Abbreviations (1 letter code)	(3 letter code)	Amino Acid		Hydrophobicity values: Kyte and Doolittle (1982)	Hydrophobicity values: Eisenberg et al., (1984b)
A	Ala	Alanine		1.8	0.62
C	Cys	Cysteine		2.5	0.29
D	Asp	Aspartic acid	(-)	-3.5	-0.90
E	Glu	Glutamic acid	(-)	-3.5	-0.74
F	Phe	Phenylalanine		2.8	1.19
G	Gly	Glycine		-0.4	0.48
H	His	Histidine	(+)	-3.2	-0.40
I	Ile	Isoleucine		4.5	1.38
K	Lys	Lysine	(+)	-3.9	-1.50
L	Leu	Leucine		3.8	1.06
M	Met	Methionine		1.9	0.64
N	Asn	Asparagine		-3.5	-0.78
P	Pro	Proline		-1.6	0.12
Q	Gln	Glutamine		-3.5	-0.85
R	Arg	Arginine	(+)	-4.5	-2.53
S	Ser	Serine		-0.8	-0.18
T	Thr	Threonine		-0.7	-0.05
V	Val	Valine		4.2	1.08
W	Trp	Tryptophan		-0.9	0.81
Y	Tyr	Tyrosine		-1.3	0.26

Appendix 3

Conservative Amino Acid Changes[1]

Ala, Ser, Thr

Asn, Gln

Asp, Glu

Ile, Leu, Met, Val

Arg, His, Lys

Phe, Trp, Tyr

[1]For the purpose of this study, amino acids on each line are considered functionally equivalent (Queen and Korn, 1984). Changes within each group are referred to as conservative changes.